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# (54) L-AMINO ACID-PRODUCING BACTERIA AND PROCESS FOR PRODUCING L-AMINO ACID

(57) An L-amino acid is produced by culturing a *Methylophilus* bacterium which can grow by using methanol as a main carbon source and has L-amino acid-producing ability, for example, a *Methylophilus* bacterium in which dihydrodipicolinate synthase activity and aspartokinase activity are enhanced by transformation through introduction into cells, of a DNA coding for dihydrodipicolinate synthase that does not suffer feedback inhibition by L-lysine and a DNA coding for aspartokinase that does not suffer feedback inhibition by L-lysine, or a *Methylophilus* bacterium made to be casamino acid auxotrophic, in a medium containing methanol as a main carbon source, to produce and accumulate an L-amino acid in culture, and collecting the L-amino acid from the culture.



EP 1 188 822 A1

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## Description

### TECHNICAL FIELD

**[0001]** The present invention relates to techniques in the field of microbial industry. In particular, the present invention relates to a method for producing an L-amino acid by fermentation, and a microorganism used in the method.

### BACKGROUND ART

**[0002]** Amino acids such as L-lysine, L-glutamic acid, L-threonine, L-leucine, L-isoleucine, L-valine and L-phenylalanine are industrially produced by fermentation by using microorganisms that belong to the genus *Brevibacterium, Corynebacterium, Bacillus, Escherichia, Streptomyces, Pseudomonas, Arthrobacter, Serratia, Penicillium, Candida* or the like. In order to improve the productivity, strains isolated from nature or artificial mutants thereof have been used as these microorganisms. Various techniques have been disclosed for enhancing activities of L-glutamic acid biosynthetic enzymes by using recombinant DNA techniques, to increase the L-glutamic acid-producing ability.

**[0003]** The productivity of L-amino acids has been considerably increased by breeding of microorganisms such as those mentioned above and the improvement of production methods. However, in order to meet further increase in the demand in future, development of methods for more efficiently producing L-amino acids at lower cost have still been desired.

**[0004]** As methods for producing amino acids by fermentation of methanol which is a fermentation raw material available in a large amount at a low cost, there have conventionally known methods using microorganisms that belong to the genus *Achromobacter* or *Pseudomonas* (Japanese Patent Publication (Kokoku) No. 45-25273/1970), *Protaminobacter* (Japanese Patent Application Laid-open (Kokai) No. 49-125590/1974), *Protaminobacter* or *Methanomonas* (Japanese Patent Application Laid-open (Kokai) No. 50-25790/1975), *Microcyclus* (Japanese Patent Application Laid-open (Kokai) No. 52-18886/1977), *Methylobacillus* (Japanese Patent Application Laid-open (Kokai) No. 4-91793/1992), *Bacillus* (Japanese Patent Application Laid-open (Kokai) No. 3-505284/1991) and so forth.

**[0005]** So far, however, no method has been known for producing L-amino acids by using *Methylophilus* bacteria. Although methods described in EP 0 035 831 A, EP 0 037 273 A and EP 0 066 994 A have been known as methods for transforming *Methylophilus* bacteria by using recombinant DNA, applying recombinant DNA techniques to improvement of amino acid productivity of *Methylophilus* bacteria has not been known.

### DISCLOSURE OF THE INVENTION

**[0006]** The object of the present invention is to provide a novel L-amino acid-producing bacterium and a method for producing an L-amino acid by using the L-amino acid-producing bacterium.

**[0007]** As a result of the present inventors' efforts devoted to achieve the aforementioned object, they found that *Methylophilus* bacteria were suitable for producing L-amino acids. Further, although it has conventionally been considered difficult to obtain auxotrophic mutants of *Methylophilus* bacteria (FEMS Microbiology Rev. 39, 235-258 (1986) and Antonie van Leeuwenhoek 53, 47-53 (1987)), the present inventors have succeeded in obtaining auxotrophic mutants of said bacteria. Thus, the present invention has been accomplished.

**[0008]** That is, the present invention provides the followings.

(1) A *Methylophilus* bacterium having L-amino acid-producing ability.
(2) The *Methylophilus* bacterium according to (1), wherein the L-amino acid is L-lysine, L-valine, L-leucine, L-isoleucine or L-threonine.
(3) The *Methylophilus* bacterium according to (1), which has resistance to an L-amino acid analogue or L-amino acid auxotrophy.
(4) The *Methylophilus* bacterium according to (1), wherein L-amino acid biosynthetic enzyme activity is enhanced.
(5) The *Methylophilus* bacterium according to (1), wherein dihydrodipicolinate synthase activity and aspartokinase activity are enhanced, and the bacterium has L-lysine-producing ability.
(6) The *Methylophilus* bacterium according to (1), wherein dihydrodipicolinate synthase activity is enhanced, and the bacterium has L-lysine-producing ability.
(7) The *Methylophilus* bacterium according to (1), wherein aspartokinase activity is enhanced, and the bacterium has L-lysine-producing ability.
(8) The *Methylophilus* bacterium according to any one of (5) to (7), wherein an activity or activities of one, two or three of enzymes selected from aspartic acid semialdehyde dehydrogenase, dihydrodipicolinate reductase and diaminopimelate decarboxylase is/are enhanced.
(9) The *Methylophilus* bacterium according to (5), wherein the dihydrodipicolinate synthase activity and the aspar-

tokinase activity are enhanced by transformation through introduction into cells, of a DNA coding for dihydrodipicolinate synthase that does not suffer feedback inhibition by L-lysine and a DNA coding for aspartokinase that does not suffer feedback inhibition by L-lysine.

(10) The bacterium according to (1), wherein activities of aspartokinase, homoserine dehydrogenase, homoserine kinase and threonine synthase, and the bacterium has L-threonine-producing ability.

(11) The bacterium according to any one of (1) to (10), wherein the *Methylophilus* bacterium is *Methylophilus methylotrophus*.

(12) A method for producing an L-amino acid, which comprises culturing a *Methylophilus* bacterium as defined in any one of the above (1) to (11) in a medium to produce and accumulate an L-amino acid in culture and collecting the L-amino acid from the culture.

(13) The method according to (12), wherein the medium contains methanol as a main carbon source.

(14) A method for producing bacterial cells of a *Methylophilus* bacterium with an increased content of an L-amino acid, which comprises culturing a *Methylophilus* bacterium as defined in any one of the above (1) to (11) in a medium to produce and accumulate an L-amino acid in bacterial cells of the bacterium.

(15) The method for producing bacterial cells of the *Methylophilus* bacterium according to (14), wherein the L-amino acid is L-lysine, L-valine, L-leucine, L-isoleucine or L-threonine.

(16) A DNA which codes for a protein defined in the following (A) or (B):

(A) a protein which has the amino acid sequence of SEQ ID NO: 6, or
(B) a protein which has an amino acid sequences of SEQ ID NO: 6 including substitution, deletion, insertion, addition or inversion of one or several amino acids, and has aspartokinase activity.

(17) The DNA according to (16), which is a DNA defined in the following (a) or (b):

(a) a DNA which has a nucleotide sequence comprising the nucleotide sequence of the nucleotide numbers 510 to 1736 of SEQ ID NO: 5; or
(b) a DNA which is hybridizable with a probe having the nucleotide sequence of the nucleotide numbers 510 to 1736 of SEQ ID NO: 5 or a part thereof under a stringent condition, and codes for a protein having aspartokinase activity.

(18) A DNA which codes for a protein defined in the following (C) or (D):

(C) a protein which has the amino acid sequence of SEQ ID NO: 8, or
(D) a protein which has an amino acid sequences of SEQ ID NO: 8 including substitution, deletion, insertion, addition or inversion of one or several amino acids, and has aspartic acid semialdehyde dehydrogenase activity.

(19) The DNA according to (18), which is a DNA defined in the following (c) or (d):

(c) a DNA which has a nucleotide sequence comprising the nucleotide sequence of the nucleotide numbers 98 to 1207 of SEQ ID NO: 7; or
(d) a DNA which is hybridizable with a probe having the nucleotide sequence of the nucleotide numbers 98 to 1207 of SEQ ID NO: 7 or a part thereof under a stringent condition, and codes for a protein having aspartic acid semialdehyde dehydrogenase activity.

(20) A DNA which codes for a protein defined in the following (E) or (F):

(E) a protein which has the amino acid sequence of SEQ ID NO: 10, or
(F) a protein which has an amino acid sequences of SEQ ID NO: 10 including substitution, deletion, insertion, addition or inversion of one or several amino acids, and has dihydrodipicolinate synthase activity.

(21) The DNA according to (20), which is a DNA defined in the following (e) or (f):

(e) a DNA which has a nucleotide sequence comprising the nucleotide sequence of the nucleotide numbers 1268 to 2155 of SEQ ID NO: 9; or
(f) a DNA which is hybridizable with a probe having the nucleotide sequence of the nucleotide numbers 1268 to 2155 of SEQ ID NO: 9 or a part thereof under a stringent condition, and codes for a protein having dihydrodipicolinate synthase activity.

(22) A DNA which codes for a protein defined in the following (G) or (H):

(G) a protein which has the amino acid sequence of SEQ ID NO: 12, or
(H) a protein which has an amino acid sequences of SEQ ID NO: 12 including substitution, deletion, insertion, addition or inversion of one or several amino acids, and has dihydrodipicolinate reductase activity.

(23) The DNA according to (22), which is a DNA defined in the following (g) or (h):

(g) a DNA which has a nucleotide sequence comprising the nucleotide sequence of the nucleotide numbers 2080 to 2883 of SEQ ID NO: 11; or
(h) a DNA which is hybridizable with a probe having the nucleotide sequence of the nucleotide numbers 2080 to 2883 of SEQ ID NO: 11 or a part thereof under a stringent condition, and codes for a protein having dihydrodipicolinate reductase activity.

(24) A DNA which codes for a protein defined in the following (I) or (J):

(I) a protein which has the amino acid sequence of SEQ ID NO: 14, or
(J) a protein which has an amino acid sequences of SEQ ID NO: 14 including substitution, deletion, insertion, addition or inversion of one or several amino acids, and has diaminopimelate decarboxylase activity.

(25) The DNA according to (24), which is a DNA defined in the following (i) or (j):

(i) a DNA which has a nucleotide sequence comprising the nucleotide sequence of the nucleotide numbers 751 to 1995 of SEQ ID NO: 13; or
(j) a DNA which is hybridizable with a probe having the nucleotide sequence of the nucleotide numbers 751 to 1995 of SEQ ID NO: 13 or a part thereof under a stringent condition, and codes for a protein having diaminopimelate decarboxylase activity.

**[0009]** In the present specification, "L-amino acid-producing ability" refers to ability to accumulate a significant amount of an L-amino acid in a medium or to increase the amino acid content in the microbial cells when a microorganism of the present invention is cultured in the medium.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0010]**

Fig. 1 shows the production process of plasmid RSF24P having a mutant *dapA*. The *"dapA*24"* refers to a mutant *dapA* that codes for a mutant DDPS wherein the 118-histidine residue is replaced with a tyrosine residue.
Fig. 2 shows the production process of plasmid RSFD80 having a mutant *dapA* and a mutant *lysC*. The *"lysC*80"* refers to a mutant *lysC* that codes for a mutant AKIII wherein the 352-threonine residue is replaced with an isoleucine residue.
Fig. 3 shows aspartokinase activity of transformant *E. coli* strains containing an *ask* gene.
Fig. 4 shows aspartic acid semialdehyde dehydrogenase activity of transformant *E. coli* strains containing an *asd* gene.
Fig. 5 shows dihydrodipicolinate synthase activity of transformant *E. coli* strains containing a *dapA* gene.
Fig. 6 shows dihydrodipicolinate reductase activity of a transformant *E. coli* strain containing a *dapB* gene.
Fig. 7 shows diaminopimelate decarboxylase activity of transformant *E. coli* strains containing a *lysA* gene.

## BEST MODE FOR CARRYING OUT THE INVENTION

<1> Microorganism of the present invention

**[0011]** The microorganism of the present invention is a bacterium belonging to the genus *Methylophilus* and having L-amino acid-producing ability. The *Methylophilus* bacterium of the present invention includes, for example, *Methylophilus methylotrophus* AS1 strain (NCIMB10515) and so forth. The *Methylophilus methylotrophus* AS1 strain (NCIMB10515) is available from National Collections of Industrial and Marine Bacteria (Address: NCIMB Lts., Torry Research Station 135, Abbey Road, Aberdeen AB9 8DG, United Kingdom).

**[0012]** L-Amino acids produced according to the present invention include L-lysine, L-glutamic acid, L-threonine, L-

valine, L-leucine, L-isoleucine, L-tryptophan, L-phenylalanine, L-tyrosine and so forth. One or more types of such amino acids may be produced.

**[0013]** *Methylophilus* bacteria having L-amino acid-producing ability can be obtained by imparting L-amino acid-producing ability to wild strains of *Methylophilus* bacteria. In order to impart L-amino acid-producing ability, there can be used methods conventionally adopted for breeding coryneform bacteria, *Escherichia* bacteria or the like, such as those methods for obtaining auxotrophic mutant strains, strains resistant to L-amino acid analogues or metabolic control mutant strains, and methods for producing recombinant strains wherein L-amino acid biosynthetic enzyme activities are enhanced (see "Amino Acid Fermentation", the Japan Scientific Societies Press [Gakkai Shuppan Center], 1st Edition, published on May 30, 1986, pp.77 to 100). In breeding of amino acid-producing bacteria, the characteristic such as auxotrophy, L-amino acid analogue resistance and metabolic control mutation may be imparted alone or in combination of two or more. The L-amino acid biosynthetic enzyme activity may be enhanced alone or in combination of two or more. Further, imparting of the characteristic such as auxotrophy, L-amino acid analogue resistance and metabolic control mutation may be combined with enhancement of the L-amino acid biosynthesis enzyme activity.

**[0014]** For example, L-lysine-producing bacteria are bred as mutants exhibiting auxotrophy for L-homoserine or L-threonine and L-methionine (Japanese Patent Publication. (Kokoku) Nos. 48-28078/1973 and 56-6499/1981), mutants exhibiting auxotrophy for inositol or acetic acid (Japanese Patent Application Laid-open (Kokai) Nos. 55-9784/1980 and 56-8692/1981), or mutants that are resistant to oxalysine, lysine hydroxamate, S-(2-aminoethyl)-cysteine, γ-methyllysine, α-chlorocaprolactam, DL-α-amino-ε-caprolactam, α-aminolauryllactam, aspartic acid analogue, sulfa drug, quinoid or N-lauroylleucine.

**[0015]** Further, L-glutamic acid-producing bacteria can be bred as mutants exhibiting auxotrophy for oleic acid or the like. L-Threonine-producing bacteria can be bred as mutants resistant to α-amino-β-hydroxyvaleric acid. L-Homoserine-producing bacteria can be bred as mutants exhibiting auxotrophy for L-threonine or mutants resistant to L-phenylalanine analogues. L-Phenylalanine-producing bacteria can be bred as mutants exhibiting auxotrophy for L-tyrosine. L-Isoleucine-producing bacteria can be bred as mutants exhibiting auxotrophy for L-leucine. L-Proline-producing bacteria can be bred as mutants exhibiting auxotrophy for L-isoleucine.

**[0016]** Furthermore, as mentioned in the examples hereinafter, strains that produce one or more kinds of branched amino acids (L-valine, L-leucine and L-isoleucine) can be obtained as strains exhibiting auxotrophy for casamino acid.

**[0017]** In order to obtain mutants from *Methylophilus* bacteria, the inventors of the present invention first examined details of an optimal mutagenesis condition by using emergence frequency of streptomycin resistant strains as an index. As a result, the maximum emergence frequency of streptomycin resistant strains was obtained when the survival rate after mutagenesis was about 0.5%, and they succeeded in obtaining auxotrophic strains under this condition. They also succeeded in obtaining auxotrophic strains, which had been considered difficult, by largely scaling up the screening of mutants compared with that previously conducted for *E. coli* and so forth.

**[0018]** As described above, since it has been revealed that mutants can be obtained by mutagenizing *Methylophilus* bacteria under a suitable condition, it has become possible to readily obtain desired mutants by suitably setting such a condition that the survival rate after the mutagenesis should become about 0.5%, depending on the mutagenesis method.

**[0019]** Mutagenesis methods for obtaining mutants from *Methylophilus* bacteria include UV irradiation and treatments with mutagenesis agents used for usual mutatagenesis treatments such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and nitrous acid. *Methylophilus* bacteria having L-amino acid-producing ability can also be obtained by selecting naturally occurring mutants of *Methylophilus* bacteria.

**[0020]** L-Amino acid analogue-resistant mutants can be obtained by, for example, inoculating mutagenized *Methylophilus* bacteria to an agar medium containing an L-amino acid analogue at a variety of concentrations and selecting strains that form colonies.

**[0021]** Auxotrophic mutants can be obtained by allowing *Methylophilus* bacteria to form colonies on an agar medium containing a target nutrient (for example, L-amino acid), replicating the colonies to an agar medium not containing said nutrient, and selecting strains that cannot grow on the agar medium not containing the nutrient.

**[0022]** Methods for imparting or enhancing L-amino acid-producing ability by enhancing L-amino acid biosynthetic enzyme activity will be exemplified below.

[L-Lysine]

**[0023]** L-Lysine-producing ability can be imparted by, for example, enhancing dihydrodipicolinate synthase activity and/or aspartokinase activity.

**[0024]** The dihydrodipicolinate synthase activity and/or the aspartokinase activity in *Methylophilus* bacteria can be enhanced by ligating a gene fragment coding for dihydrodipicolinate synthase and/or a gene fragment coding for aspartokinase with a vector that functions in *Methylophilus* bacteria, preferably a multiple copy type vector, to create a recombinant DNA, and introducing them into a *Methylophilus* bacterium host to transform the host. As a result of the

increase in the copy numbers of the gene coding for dihydrodipicolinate synthase and/or the gene coding for aspartokinase in cells of the transformant strain, the activity or activities thereof is/are enhanced. Hereafter, dihydrodipicolinate synthase, aspartokinase and aspartokinase III are also referred with abbreviations of DDPS, AK and AKIII, respectively.

**[0025]** As a microorganism providing a gene that codes for DDPS and a gene that codes for AK, any microorganisms can be used so long as they have genes enabling expression of DDPS activity and AK activity in microorganisms belonging to the genus *Methylophilus*. Such microorganisms may be wild strains or mutant strains derived therefrom. Specifically, examples of such microorganisms include *E. coli* (*Escherichia coli*) K-12 strain, *Methylophilus methylotrophus* AS1 strain (NCIMB10515) and so forth. Since nucleotide sequences of a gene coding for DDPS *(dapA,* Richaud, F. et al., J. Bacteriol., 297, (1986)) and a gene coding for AKIII (*lysC,* Cassan, M., Parsot, C., Cohen, G.N. and Patte, J.C., J. Biol. Chem., 261, 1052 (1986)) derived from *Escherichia* bacteria have been both revealed, these genes can be obtained by PCR using primers synthesized based on the nucleotide sequences of these genes and chromosome DNA of microorganism such as *E. coli* K-12 or the like as a template. As specific examples, *dapA* and *lysC* derived from *E. coli* will be explained below. However, genes used for the present invention are not limited to them.

**[0026]** It is preferred that DDPS and AK used for the present invention do not suffer feedback inhibition by L-lysine. It has been known that wild-type DDPS derived from *E. coli* suffers feedback inhibition by L-lysine, and that wild-type AKIII derived from *E. coli* suffers suppression and feedback inhibition by L-lysine. Therefore, *dapA* and *lysC* to be introduced into *Methylophilus* bacteria preferably code for DDPS and AKIII having a mutation that desensitizes the feedback inhibition by L-lysine. Hereafter, DDPS having a mutation that desensitizes the feedback inhibition by L-lysine is also referred to as "mutant DDPS", and DNA coding for the mutant DDPS is also referred to as "mutant *dapA*". AKIII derived from *E. coli* having a mutation that desensitizes the feedback inhibition by L-lysine is also referred to as "mutant AKIII", and DNA coding for the mutant AKIII is also referred to as "mutant *lysC*".

**[0027]** According to the present invention, DDPS and AK are not necessarily required to be a mutant. It has been known that, for example, DDPS derived from *Corynebacterium* bacteria originally does not suffer feedback inhibition by L-lysine.

**[0028]** A nucleotide sequence of wild-type *dapA* derived from *E. coli* is exemplified by SEQ ID NO: 1. The amino acid sequence of wild-type DDPS coded by said nucleotide sequence is exemplified by SEQ ID NO: 2. A nucleotide sequence of wild-type *lysC* derived from *E. coli* is exemplified by SEQ ID NO: 3. The amino acid sequence of wild-type ATIII coded by said nucleotide sequence is exemplified by SEQ ID NO: 4.

**[0029]** The DNA coding for mutant DDPS that does not suffer feedback inhibition by L-lysine includes a DNA coding for DDPS having the amino acid sequence described in SEQ ID NO: 2 wherein the 118-histidine residue is replaced with a tyrosine residue. The DNA coding for mutant AKIII that does not suffer feedback inhibition by L-lysine includes a DNA coding for AKIII having an amino sequence described in SEQ ID NO: 4 wherein the 352-threonine residue is replaced with an isoleucine residue.

**[0030]** The plasmid used for gene cloning may be any plasmid so long as it can replicate in microorganisms such as *Escherichia* bacteria or the like, and specifically include pBR322, pTWV228, pMW119, pUC19 and so forth.

**[0031]** The vector that functions in *Methylophilus* bacteria is, for example, a plasmid that can autonomously replicate in *Methylophilus* bacteria. Specifically, there can be mentioned RSF1010, which is a broad host spectrum vector, and derivatives thereof, for example, pAYC32 (Chistorerdov, A.Y., Tsygankov, Y.D. Plasmid, 16, 161-167, (1986)), pMFY42 (Gene, 44, 53, (1990)), pRP301, pTB70 (Nature, 287, 396, (1980)) and so forth.

**[0032]** In order to prepare a recombinant DNA by ligating *dapA* and *lysC* to a vector that functions in *Methylophilus* bacteria, the vector is digested with a restriction enzyme that corresponds to the terminus of DNA fragment containing *dapA* and *lysC*. Ligation is usually performed by using ligase such as T4 DNA ligase. *dapA* and *lysC* may be individually incorporated into separate vectors or into a single vector.

**[0033]** As a plasmid containing a mutant *dapA* coding for mutant DDPS and a mutant *lysC* coding for mutant AKIII, a broad host spectrum plasmid RSFD80 has been known (WO95/16042). *E. coli* JM109 strain transformed with this plasmid was designated as AJ12396, and deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (postal code 305-8566, 1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on October 28, 1993 and received an accession number of FERM P-13936, and it was transferred to an international deposition under the provisions of the Budapest Treaty on November 1, 1994, and received an accession number of FERM BP-4859. RSFD80 can be obtained from the AJ12396 strain in a known manner.

**[0034]** The mutant *dapA* contained in RSFD80 has a nucleotide sequence of wild-type *dapA* of SEQ ID NO: 1 including replacement of C at the nucleotide number 597 with T. The mutant DDPS encoded thereby has an amino acid sequence of SEQ ID NO: 2 including replacement of the 118-histidine residue with a tyrosine residue. The mutant *lysC* contained in RSFD80 has a nucleotide sequence of wild-type *lysC* of SEQ ID NO: 3 including replacement of C at the nucleotide number 1638 with T. The mutant AKIII encoded thereby has an amino acid sequence of SEQ ID NO: 4 including replacement of the 352-threonine residue with an isoleucine residue.

**[0035]** In order to introduce a recombinant DNA prepared as described above into *Methylophilus* bacteria, any method can be used so long as it provides sufficient transformation efficiency. For example, electroporation can be used (Canadian Journal of Microbiology, 43, 197 (1997)).

**[0036]** The DDPS activity and/or the AK activity can also be enhanced by the presence of multiple copies of *dapA* and/or *lysC* on chromosome DNA of *Methylophilus* bacteria. In order to introduce multiple copies of *dapA* and/or *lysC* into chromosome DNA of *Methylophilus* bacteria, homologous recombination is performed by using, as a target, a sequence that is present on chromosome DNA of *Methylophilus* bacteria in a multiple copy number. As the sequence present on chromosome DNA in the multiple copy number, a repetitive DNA, inverted repeats present at the end of a transposable element, or the like can be used. Alternatively, as disclosed in Japanese Patent Application Laid-open (Kokai) No. 2-109985/1990, multiple copies of *dapA* and/or *lysC* can be introduced into chromosome DNA by mounting them on a transposon to transfer them. In both of the methods, as a result of increased copy number of *dapA* and/or *lysC* in transformed strains, the DDPS activity and the AK activity should be amplified.

**[0037]** Besides the above gene amplification, the DDPS activity and/or the AK activity can be amplified by replacing an expression control sequence such as promoters of *dapA* and/or *lysC* with stronger ones (Japanese Patent Application Laid-open (Kokai) No. 1-215280/1989). As such strong promoters, there have been known, for example, *lac* promoter, *trp* promoter, *trc* promoter, *tac* promoter, $P_R$ promoter and $P_L$ promoter of lambda phage, tet promoter, *amyE* promoter, *spac* promoter and so forth. Substitution of these promoters enhances expression of *dapA* and/or *lysC*, and thus the DDPS activity and the AK activity are amplified. Enhancement of expression control sequences can be combined with increase of the copy numbers of *dapA* and/or *lysC*.

**[0038]** In order to prepare a recombinant DNA by ligating a gene fragment and a vector, the vector is digested with a restriction enzyme corresponding to the terminus of the gene fragment. Ligation is usually performed by ligase such as T4 DNA ligase. As methods for digestion, ligation and others of DNA, preparation of chromosome DNA, PCR, preparation of plasmid DNA, transformation, design of oligonucleotides used as primers and so forth, usual methods well known to those skilled in the art can be used. Such methods are described in Sambrook, J., Fritsch, E. F., and Maniatis, T., "Molecular Cloning: A Laboratory Manual, 2nd Edition", Cold Spring Harbor Laboratory Press, (1989) and so forth.

**[0039]** In addition to the enhancement of the DDPS activity and/or the AK activity, activity of another enzyme involved in the L-lysine biosynthesis may also be enhanced. Such enzymes include diaminopimelate pathway enzymes such as dihydrodipicolinate reductase, diaminopimelate decarboxylase, diaminopimelate dehydrogenase (WO96/40934 for all of the foregoing enzymes), phosphoenolpyruvate carboxylase (Japanese Patent Application Laid-open (Kokai) No. 60-87788/1985), aspartate aminotransferase (Japanese Patent Publication (Kokoku) No. 6-102028/1994), diaminopimelate epimerase, aspartic acid semialdehyde dehydrogenase and so forth, or aminoadipate pathway enzymes such as homoaconitate hydratase and so forth. Preferably, activity of at least one enzyme of aspartic acid semialdehyde dehydrogenase, dihydrodipicolinate reductase and diaminopimelate decarboxylase is enhanced.

**[0040]** Aspartokinase, aspartic acid semialdehyde dehydrogenase, dihydrodipicolinate synthase, dihydrodipicolinate reductase and diaminopimelate decarboxylase derived form *Methylophilus methylotrophus* will be described later.

**[0041]** Further, the microorganisms of the present invention may be decreased in activity of an enzyme that catalyzes a reaction for generating a compound other than L-lysine by branching off from the biosynthetic pathway of L-lysine, or may be deficient in such an enzyme. The enzyme that catalyzes the reaction for generating the compound other than L-lysine by branching off from the biosynthetic pathway L-lysine include homoserine dehydrogenase (see WO95/23864).

**[0042]** The aforementioned techniques for enhancing activity of an enzyme involved in the L-lysine biosynthesis can be similarly used for other amino acids mentioned below.

[L-Glutamic acid]

**[0043]** L-Glutamic acid-producing ability can be imparted to *Methylophilus* bacteria by, for example, introducing a DNA that codes for any one of enzymes including glutamate dehydrogenase (Japanese Patent Application Laid-open (Kokai) 61-268185/1986), glutamine synthetase, glutamate synthase, isocitrate dehydrogenase (Japanese Patent Application Laid-open (Kokai) Nos. 62-166890/1987 and 63-214189/1988), aconitate hydratase (Japanese Patent Application Laid-open (Kokai) No. 62-294086/1987), citrate synthase (Japanese Patent Application Laid-open (Kokai) Nos. 62-201585/1987 and 63-119688/1988), phosphoenolpyruvate carboxylase (Japanese Patent Application Laid-open (Kokai) Nos. 60-87788/1985 and 62-55089/1987), pyruvate dehydrogenase, pyruvate kinase, phosphoenolpyruvate synthase, enolase, phosphoglyceromutase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, triose phosphate isomerase, fructose bisphosphate aldolase, phosphofructokinase (Japanese Patent Application Laid-open (Kokai) No. 63-102692/1988), glucose phosphate isomerase, glutamine-oxoglutarate aminotransferase (WO99/07853) and so forth.

**[0044]** Further, the microorganisms of the present invention may be decreased in activity of an enzyme that catalyzes

a reaction for generating a compound other than L-glutamic acid by branching off from the biosynthetic pathway of L-glutamic acid, or may be deficient in such an enzyme. The enzyme that catalyzes the reaction for generating the compound other than L-glutamic acid by branching off from the biosynthetic pathway L-glutamic acid include α-ketoglutarate dehydrogenase (αKGDH), isocitrate lyase, phosphate acetyltransferase, acetate kinase, acetohydroxy acid synthase, acetolactate synthase, formate acetyltransferase, lactate dehydrogenase, glutamate decarboxylase, 1-pyrroline dehydrogenase and so forth.

[L-Threonine]

**[0045]** L-Threonine-producing ability can be imparted or enhanced by, for example, enhancing activities of aspartokinase, homoserine dehydrogenase, homoserine kinase and threonine synthase. The activities of these enzymes can be enhanced by, for example, transforming *Methylophilus* bacteria using a recombinant plasmid containing a threonine operon (Japanese Patent Application Laid-open (Kokai) Nos. 55-131397/1980, 59-31691/1984 and 56-15696/1981 and Japanese Patent Application Laid-open (Kohyo) No. 3-501682/1991).

**[0046]** The production ability can also be imparted or enhanced by amplifying or introducing a threonine operon having a gene coding for aspartokinase of which feedback inhibition by L-threonine is desensitized (Japanese Patent Publication (Kokoku) No. 1-29559/1989), a gene coding for homoserine dehydrogenase (Japanese Patent Application Laid-open (Kokai) No. 60-012995/1985) or a gene coding for homoserine kinase and homoserine dehydrogenase (Japanese Patent Application Laid-open (Kokai) No. 61-195695/1986).

**[0047]** Further, L-threonine-producing ability can be improved by introducing a DNA coding for a mutant phosphoenolpyruvate carboxylase having a mutation for desensitizing feedback inhibition by aspartic acid.

[L-Valine]

**[0048]** L-Valine-producing ability can be imparted by, for example, introducing into *Methylophilus* bacteria an L-valine biosynthesis gene whose control mechanism has been substantially desensitized. There may also be introduced a mutation that substantially desensitizes a control mechanism of an L-valine biosynthesis gene carried by a microorganism belonging to the genus *Methylophilus.*

**[0049]** Examples of the L-valine biosynthesis gene include, for example, the *ilvGMEDA* operon of *E. coli.* Threonine deaminase encoded by an *ilvA* gene catalyzes the deamination reaction converting L-threonine into 2-ketobutyric acid, which is the rate-determining step of L-isoleucine biosynthesis. Therefore, in order to attain efficient progression of the L-valine synthesis reactions, it is preferable to use an operon that does not express threonine deaminase activity. Examples of the *ilvGMEDA* operon that does not express such threonine deaminase activity include an *ilvGMEDA* operon wherein a mutation for eliminating threonine deaminase activity is introduced into *ilvA,* or *ilvA* is disrupted, and an *ilvGMED* operon wherein *ilvA* is deleted.

**[0050]** Since the *ilvGMEDA* operon suffers expression control of operon (attenuation) by L-valine and/or L-isoleucine and/or L-leucine, the region required for the attenuation is preferably removed or mutated to desensitize the suppression of expression by L-valine.

**[0051]** An *ilvGMEDA* operon which does not express threonine deaminase activity and in which attenuation is desensitized as described above can be obtained by subjecting a wild-type *ilvGMEDA* operon to a mutagenesis treatment or modifying it by means of gene recombination techniques (see WO96/06926).

[L-Leucine]

**[0052]** L-Leucine-producing ability is imparted or enhanced by, for example, introducing into a microorganism belonging to the genus *Methylophilus* an L-leucine biosynthesis gene whose control mechanism has been substantially desensitized, in addition to the above characteristics required for the production of L-valine. It is also possible to introduce such a mutation that the control mechanism of an L-leucine biosynthesis gene in a microorganism belonging to the genus *Methylophilus* should be substantially eliminated. Examples of such a gene include, for example, an *leuA* gene which provides an enzyme in which inhibition by L-leucine is substantially eliminated.

[L-Isoleucine]

**[0053]** L-Isoleucine-producing ability can be imparted by, for example, introducing a *thrABC* operon containing a *thrA* gene coding for aspartokinase I/homoserine dehydrogenase I derived from *E. coli* wherein inhibition by L-threonine has been substantially desensitized and an *ilvGMEDA* operon which contains an *ilvA* gene coding for threonine deaminase wherein inhibition by L-isoleucine is substantially desensitized and whose region required for attenuation is removed (Japanese Patent Application Laid-open (Kokai) No. 8-47397/1996).

[Other amino acids]

**[0054]** Biosyntheses of L-tryptophan, L-phenylalanine, L-tyrosine, L-threonine and L-isoleucine can be enhanced by increasing phosphoenolpyruvate-producing ability of *Methylophilus* bacteria (WO97/08333).

**[0055]** The production abilities for L-phenylalanine and L-tyrosine are improved by amplifying or introducing a desensitized chorismate mutase-prephenate dehydratase (CM-PDT) gene (Japanese Patent Application Laid-open (Kokai) Nos. 5-236947/1993 and 62-130693/1987) and a desensitized 3-deoxy-D-arabinoheptulonate-7-phosphate synthase (DS) gene (Japanese Patent Application Laid-open (Kokai) Nos. 5-236947/1993 and 61-124375/1986).

**[0056]** The producing ability of L-tryptophan is improved by amplifying or introducing a tryptophan operon containing a gene coding for desensitized anthranilate synthase (Japanese Patent Application Laid-open (Kokai) Nos. 57-71397/1982, 62-244382/1987 and US Patent No. 4,371,614).

**[0057]** In the present specification, the expression that enzyme "activity is enhanced" usually refers to that the intracellular activity of the enzyme is higher than that of a wild type strain, and when a strain in which the activity of the enzyme is enhanced is obtained by modification using gene recombinant techniques or the like, the intracellular activity of the enzyme is higher than that of the strain before the modification. The expression that enzyme "activity is decreased" usually refers to that the intracellular activity of the enzyme is lower than that of a wild type strain, and when a strain in which the activity of the enzyme is decreased is obtained by modification using gene recombinant techniques or the like, the intracellular activity of the enzyme is lower than that of the strain before the modification.

**[0058]** L-Amino acids can be produced by culturing *Methylophilus* bacteria having L-amino acid-producing ability obtained as described above in a medium to produce and accumulate L-amino acids in the culture, and collecting the L-amino acids from the culture.

**[0059]** Bacterial cells of *Methylophilus* bacteria with an increased L-amino acid content compared with wild strains of *Methylophilus* bacteria can be produced by culturing *Methylophilus* bacteria having L-amino acid-producing ability in a medium to produce and accumulate L-amino acids in bacterial cells of the bacteria.

**[0060]** Microorganisms used for the present invention can be cultured by methods usually used for culturing microorganisms having methanol-assimilating property. The medium used for the present invention may be a natural or synthetic medium so long as it contains a carbon source, a nitrogen source, inorganic ions and other trace amount organic constituents as required.

**[0061]** By using methanol as a main carbon source, L-amino acids can be prepared at a low cost. When methanol is used as a main carbon source, it is usually added to a medium in an amount of 0.001 to 30%. As the nitrogen source, ammonium sulfate or the like is used by adding it to the medium. Other than these, there are usually added small amounts of the trace amount constituents such as potassium phosphate, sodium phosphate, magnesium sulfate, ferrous sulfate and manganese sulfate.

**[0062]** The culture is usually performed under an aerobic condition obtained by, for example, shaking or stirring for aeration, at pH 5 to 9 and a temperature of 20 to 45°C, and it is usually completed within 24 to 120 hours.

**[0063]** Collection of L-amino acids from culture can be usually attained by a combination of known methods such as those using ion exchange resin, precipitation and others.

**[0064]** Further, *Methylophilus* bacterium cells can be separated from the medium by usual methods for separating microbial cells.

<2> Gene of the present invention

**[0065]** The DNA of the present invention is a gene which codes for one of the enzymes, aspartokinase (henceforth also abbreviated as "AK"), aspartic acid semialdehyde dehydrogenase (henceforth also abbreviated as "ASD"), dihydrodipicolinate synthase (henceforth also abbreviated as "DDPS"), dihydrodipicolinate reductase (henceforth also abbreviated as "DDPR"), and diaminopimelate decarboxylase (henceforth also abbreviated as "DPDC") derived from *Methylophilus methylotrophus*.

**[0066]** The DNA of the present invention can be obtained by, for example, transforming a mutant strain of a microorganism deficient in AK, ASD, DDPS, DDPR or DPDC using a gene library of *Methylophilus methylotrophus*, and selecting a clone in which auxotrophy is recovered.

**[0067]** A gene library of *Methylophilus methylotrophus* can be produced as follows, for example. First, total chromosome DNA is prepared from a *Methylophilus methylotrophus* wild strain, for example, the *Methylophilus methylotrophus* AS1 strain (NCIMB10515), by the method of Saito et al. (Saito, H. and Miura, K., Biochem. Biophys. Acta 72, 619-629, (1963)) or the like, and partially digested with a suitable restriction enzyme, for example, *Sau*3AI or *Alu*I, to obtain a mixture of various fragments. By controlling the degree of the digestion through adjustment of digestion reaction time and so forth, a wide range of restriction enzymes can be used.

**[0068]** Subsequently, the digested chromosome DNA fragments are ligated to vector DNA autonomously replicable in *Escherichia coli* cells to produce recombinant DNA. Specifically, a restriction enzyme producing the same terminal

nucleotide sequence as that produced by the restriction enzyme used for the digestion of chromosome DNA is allowed to act on the vector DNA to fully digest and cleave the vector. Then, the mixture of chromosome DNA fragments and the digested and cleaved vector DNA are mixed, and a ligase, preferably T4 DNA ligase, is allowed to act on the mixture to obtain recombinant DNA.

**[0069]** A gene library solution can be obtained by transforming *Escherichia coli,* for example, the *Escherichia coli* JM109 strain or the like, using the obtained recombinant DNA, and preparing recombinant DNA from the culture broth of the transformant. This transformation can be performed by the method of D.M. Morrison (Methods in Enzymology 68, 326 (1979)), the method of treating recipient cells with calcium chloride so as to increase the permeability of DNA (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)) and so forth. In the examples mentioned hereinafter, electroporation was used.

**[0070]** As examples of the aforementioned vector, there can be mentioned pUC19, pUC18, pUC118, pUC119, pBR322, pHSG299, pHSG298, pHSG399, pHSG398, RSF1010, pMW119, pMW118, pMW219, pMW218, pSTV28, pSTV29 and so forth. Phage vectors can also be used. Since pUC118 and pUC119 contain an ampicillin resistance gene, and pSTV28 and pSTV29 contain a chloramphenicol resistance gene, for example, only transformants which harbor a vector or a recombinant DNA can be grown by using a medium containing ampicillin or chloramphenicol.

**[0071]** As the method for culturing the transformants and collecting recombinant DNA from bacterial cells, the alkali SDS method and the like can be mentioned.

**[0072]** A mutant microbial strain deficient in AK, ASD, DDPS, DDPR or DPDC is transformed by using the gene library solution of *Methylophilus methylotrophus* obtained as described above, and clones whose auxotrophy is recovered are selected.

**[0073]** Examples of a mutant microbial strain deficient in AK include *E. coli* GT3 deficient in three kinds of genes coding for AK (*thrA, metLM, lysC*). Examples of a mutant microbial strain deficient in ASD include *E. coli* Hfr3000 U482 (CGSC 5081 strain). Examples of a mutant microbial strain deficient in DDPS include *E. coli* AT997 (CGSC 4547 strain). Examples of a mutant microbial strain deficient in DDPR include *E. coli* AT999 (CGSC 4549 strain). Examples of a mutant microbial strain deficient in DPDC include *E. coli* AT2453 (CGSC 4505 strain). These mutant strains can be obtained from *E. coli* Genetic Stock Center (the Yale University, Department of Biology, Osborn Memorial Labs., P.O. Box 6666, New Haven 06511-7444, Connecticut, U.S.).

**[0074]** Although all of the aforementioned mutant strains cannot grow in M9 minimal medium, transformant strains which contain a gene coding for AK, ASD, DDPS, DDPR or DPDC can grow in M9 minimal medium because these genes function in the transformants. Therefore, by selecting transformant strains that can grow in the minimal medium and collecting recombinant DNA from the strains, DNA fragments containing a gene that codes for each enzyme can be obtained. *E. coli* AT999 (CGSC 4549 strain) shows extremely slow growth rate even in a complete medium such as L medium when diaminopimelic acid is not added to the medium. However, normal growth can be observed for its transformant strains which contain a gene coding for DDPR derived from *Methylophilus methylotrophus,* because of the function of the gene. Therefore, a transformant strain that contains a gene coding for DDPR can also be obtained by selecting a transformant strain normally grown in L medium.

**[0075]** By extracting an insert DNA fragment from the obtained recombinant DNA and determining its nucleotide sequence, an amino acid sequence of each enzyme and nucleotide sequence of the gene coding for it can be determined.

**[0076]** The gene coding for AK of the present invention (henceforth also referred to *"ask"*) codes for AK which has the amino acid sequence of SEQ ID NO: 6 shown in Sequence Listing. As a specific example of the *ask* gene, there can be mentioned a DNA having the nucleotide sequence which consists of nucleotides of SEQ ID NO: 5. The *ask* gene of the present invention may have a sequence in which codon corresponding to each of the amino acids is replaced with equivalent codon so long as it codes for the same amino acid sequence as the amino acid sequence of SEQ ID NO: 6.

**[0077]** The gene which codes for ASD of the present invention (henceforth also referred to as *"asd")* codes for ASD which has the amino acid sequence of SEQ ID NO: 8 shown in Sequence Listing. As a specific example of the *asd* gene, a DNA which contains the nucleotide sequence consisting of the nucleotides of the nucleotide numbers 98-1207 in SEQ ID NO: 7 can be mentioned. The *asd* gene of the present invention may have a sequence in which codon corresponding to each of the amino acids is replaced with equivalent codon so long as it codes for the same amino acid sequence as the amino acid sequence of SEQ ID NO: 8.

**[0078]** The gene which codes for DDPS of the present invention (henceforth also referred to as *"dapA"*) codes for DDPS which has the amino acid sequence of SEQ ID NO: 10 shown in Sequence Listing. As a specific example of the *dapA* gene, a DNA which has the nucleotide sequence consisting of the nucleotides of the nucleotide numbers 1268-2155 in SEQ ID NO: 9 can be mentioned. The *dapA* gene of the present invention may have a sequence in which codon corresponding to each of the amino acids is replaced with equivalent codon so long as it codes for the same amino acid sequence as the amino acid sequence of SEQ ID NO: 10.

**[0079]** The gene which codes for DDBR of the present invention (henceforth also referred to as *"dapB")* codes for

DDBR which has the amino acid sequence of SEQ ID NO: 12 shown in Sequence Listing. As a specific example of the *dapB* gene, a DNA which has the nucleotide sequence consisting of the nucleotides of the nucleotide numbers 2080-2883 in SEQ ID NO: 11 can be mentioned. The *dapB* gene of the present invention may have a sequence in which codon corresponding to each of the amino acids is replaced with equivalent codon so long as it codes for the same amino acid sequence as the amino acid sequence of SEQ ID NO: 12.

**[0080]** The gene which codes for DPDC of the present invention (henceforth also referred to as "*lysA*") codes for DPDC which has the amino acid sequence of SEQ ID NO: 14 shown in Sequence Listing. As a specific example of the *lysA* gene, a DNA which has the nucleotide sequence consisting of the nucleotides of the nucleotide numbers 751-1995 in SEQ ID NO: 13 can be mentioned. The *lysA* gene of the present invention may have a sequence in which codon corresponding to each of the amino acids is replaced with equivalent codon so long as it codes for the same amino acid sequence as the amino acid sequence of SEQ ID NO: 14.

**[0081]** The gene for each enzyme of the present invention may have an amino acid sequence corresponding to each amino acid sequence of SEQ ID NO: 6, 8, 10, 12 or 14 including substitution, deletion, insertion, addition or inversion of one or several amino acids, and may code a protein having activity of AK, ASD, DDPS, DDPR or DPDC. The expression "one or several" used herein preferably means a number of 1 to 10, more preferably a number of 1 to 5, more preferably a number of 1 to 2.

**[0082]** The DNA which codes for the substantially same protein as AK, ASD, DDPS, DDPR or DPDC such as those mentioned above can be obtained by modifying each nucleotide sequence so that the amino acid sequence should contain substitution, deletion, insertion, addition or inversion of an amino acid residue or residues at a particular site by, for example, site-specific mutagenesis. Such a modified DNA as mentioned above may also be obtained by a conventional mutagenesis treatment. Examples of the mutagenesis treatment include in vitro treatment of DNA coding for AK, ASD, DDPS, DDPR or DPDC with hydroxylamine or the like, treatment of a microorganism such as *Escherichia* bacteria containing a gene coding for AK, ASD, DDPS, DDPR or DPDC by UV irradiation or with mutagenesis agents used for usual mutagenesis treatment such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and nitrous acid.

**[0083]** The aforementioned substitution, deletion, insertion, addition or inversion of nucleotides includes naturally occurring mutations (mutant or variant) such as those observed depending difference between species or strains of microorganisms containing AK, ASD, DDPS, DDPR or DPDC and so forth.

**[0084]** The DNA which codes for substantially the same protein as AK, ASD, DDPS, DDPR or DPDC can be obtained by allowing expression of a DNA having such a mutation as mentioned above in a suitable cell, and examining AK, ASD, DDPS, DDPR or DPDC activity of the expression product. The DNA which codes for substantially the same protein as AK, ASD, DDPS, DDPR or DPDC can also be obtained by isolating, from DNAs coding for AK, ASD, DDPS, DDPR or DPDC which have mutations or cells containing each of them, a DNA hybridizable with a probe containing a nucleotide sequence comprising the nucleotide sequence of the nucleotide numbers 510-1736 of SEQ ID NO: 5, a nucleotide sequence comprising the nucleotide sequence of the nucleotide numbers 98-1207 of SEQ ID NO: 7, a nucleotide sequence comprising the nucleotide sequence of the nucleotide numbers 1268-2155 of SEQ ID NO: 9, a nucleotide sequence comprising the nucleotide sequence of the nucleotide numbers 2080-2883 of SEQ ID NO: 11, or a nucleotide sequence comprising the nucleotide sequence of the nucleotide numbers 751-1995 of SEQ ID NO: 13, or a part of those nucleotide sequences under a stringent condition, and coding for a protein having AK, ASD, DDPS, DDPR or DPDC activity. In the present specification, to have a nucleotide sequence or a part thereof means to have the nucleotide sequence or the part thereof, or a nucleotide complementary thereto.

**[0085]** The term "stringent condition" used herein means a condition that allows formation of so-called specific hybrid and does not allow formation of non-specific hybrid. This condition may vary depending on the nucleotide sequence and length of the probe. However, it may be, for example, a condition that allows hybridization of highly homologous DNA such as DNA having homology of 40% or higher, but does not allow hybridization of DNA of lower homology than defined above, or a condition that allows hybridization under a washing condition of usual Southern hybridization, of a temperature of 60°C and salt concentrations corresponding to 1 x SSC and 0.1% SDS, preferably 0.1 x SSC and 0.1% SDS.

**[0086]** A partial sequence of each gene can also be used as the probe. Such a probe can be produced by PCR (polymerase chain reaction) using oligonucleotides produced based on a nucleotide sequence of each gene as primers and a DNA fragment containing each gene as a template. When a DNA fragment having a length of about 300 bp is used as the probe, washing condition for hybridization may be, for example, 50°C, 2 x SSC and 0.1% SDS.

**[0087]** Genes that hybridize under such a condition as mentioned above also include those having a stop codon occurring in its sequence and those encoding an enzyme no longer having its activity due to a mutation of active center. However, such genes can readily be eliminated by ligating the genes to a commercially available activity expression vector, and measuring AK, ASD, DDPS, DDPR or DPDC activity.

**[0088]** Since the nucleotide sequences of the genes that codes for AK, ASD, DDPS, DDPR and DPDC derived from *Methylophilus methylotrophus* were revealed by the present invention, DNA sequences which code for AK, ASD, DDPS, DDPR and DPDC can be obtained from a *Methylophilus methylotrophus* gene library by hybridization using oligonu-

cleotide probes produced based on the sequences. Moreover, DNA sequences which code for these enzymes can also be obtained by amplifying them from *Methylophilus methylotrophus* chromosome DNA by PCR using oligonucleotide primers produced based on the aforementioned nucleotide sequences.

**[0089]** The aforementioned genes can suitably be utilized to enhance L-lysine-producing ability of *Methylophilus* bacteria.

EXAMPLES

**[0090]** The present invention will further specifically be explained with reference to the following examples hereafter.

**[0091]** The reagents used were obtained from Wako Pure Chemicals or Nakarai Tesque unless otherwise indicated. The compositions of the media used in each example are shown below. pH was adjusted with NaOH or HCl for all media.

| (L medium) | |
|---|---|
| Bacto trypton (DIFCO) | 10 g/L |
| Yeast extract (DIFCO) | 5 g/L |
| NaCl | 5 g/L |
| [steam-sterilized at 120°C for 20 minutes] | |

| (L agar medium) | |
|---|---|
| L medium | |
| Bacto agar (DIFCO) | 15 g/L |
| [steam-sterilized at 120°C for 20 minutes] | |

| (SOC medium) | |
|---|---|
| Bacto trypton (DIFCO) | 20 g/L |
| Yeast extract (DIFCO) | 5 g/L |
| 10 mM NaCl | |
| 2.5 mM KCl | |
| 10 mM $MgSO_4$ | |
| 10 mM $MgCl_2$ | |
| 20 mM Glucose | |

[The constituents except for magnesium solution and glucose were steam-sterilized (120°C, 20 minutes), then 2 M magnesium stock solution (1 M $MgSO_4$, 1 M $MgCl_2$) and 2 M glucose solution, which solutions had been passed through a 0.22-μm filter, were added thereto, and the mixture was passed through a 0.22-μm filter again.]

| (121M1 medium) | |
|---|---|
| $K_2HPO_4$ | 1.2 g/L |
| $KH_2PO_4$ | 0.62 g/L |
| NaCl | 0.1 g/L |
| $(NH_4)_2SO_4$ | 0.5 g/L |
| $MgSO_4 \cdot 7H_2O$ | 0.2 g/L |
| $CaCl_2 \cdot 6H_2O$ | 0.05 g/L |
| $FeCl_3 \cdot 6H_2O$ | 1.0 mg/L |
| $H_3BO_3$ | 10 μg/L |
| $CuSO_4 \cdot 5H_2O$ | 5 μg/L |
| $MnSO_4 \cdot 5H_2O$ | 10 μg/L |
| $ZnSO_4 \cdot 7H_2O$ | 70 μg/L |
| $NaMoO_4 \cdot 2H_2O$ | 10 μg/L |

(continued)

| (121M1 medium) | |
|---|---|
| $CoCl_2 \cdot 6H_2O$ | 5 μg/L |
| Methanol 1% (vol/vol), pH 7.0 | |

[The constituents except for methanol were steam-sterilized at 121°C for 15 minutes. After the constituents sufficiently cooled, methanol was added.]

| (Composition of 121 production medium) | |
|---|---|
| Methanol | 2% |
| Dipotassium phosphate | 0.12% |
| Potassium phosphate | 0.062% |
| Calcium chloride hexahydrate | 0.005% |
| Magnesium sulfate heptahydrate | 0.02% |
| Sodium chloride | 0.01% |
| Ferric chloride hexahydrate | 1.0 mg/L |
| Ammonium sulfate | 0.3% |
| Cupric sulfate pentahydrate | 5 μg/L |
| Manganous sulfate pentahydrate | 10 μg/L |
| Sodium molybdate dihydrate | 10 μg/L |
| Boric acid | 10 μg/L |
| Zinc sulfate heptahydrate | 70 μg/L |
| Cobaltous chloride hexahydrate | 5 μg/L |
| Calcium carbonate (Kanto Kagaku) | 3% |
| (pH 7.0) | |

| (121M1 Agar medium) | |
|---|---|
| 121M1 medium | |
| Bacto agar (DIFCO) | 15 g/L |

[The constituents except for methanol were steam-sterilized at 121°C for 15 minutes. After the constituents sufficiently cooled, methanol was added.]

| (M9 minimal medium) | |
|---|---|
| $Na_2HPO_4 \cdot 12H_2O$ | 16 g/L |
| $KH_2PO_4$ | 3 g/L |
| NaCl | 0.5 g/L |
| $NH_4Cl$ | 1 g/L |
| $MgSO_4 \cdot 7H_2O$ | 246.48 mg/L |
| Glucose | 2 g/L |
| pH 7.0 | |

[$MgSO_4$ and glucose were separately sterilized (120°C, 20 minutes) and added. A suitable amount of amino acids and vitamins were added as required.]

| (M9 minimal agar medium) | |
|---|---|
| M9 minimal medium | |
| Bacto agar (DIFCO) | 15 g/L |

Example 1

Creation of L-lysine-producing bacterium (1)

(1) Introduction of mutant *lysC* and mutant *dapA* into *Methylophilus* bacterium

**[0092]** A mutant *lysC* and a mutant *dapA* were introduced into a *Methylophilus* bacterium by using a known plasmid RSFD80 (see WO95/16042) containing them. RSFD80 is a plasmid pVIC40 (International Publication WO90/04636, Japanese Patent Application Laid-open (Kohyo) No. 3-501682/1991) derived from a broad host spectrum vector plasmid pAYC32 (Chistorerdov, A.Y., Tsygankov, Y.D., Plasmid, 16, 161-167, (1986)), which is a derivative of RSF1010, in which a mutant *dapA* and a mutant *lysC* derived from *E. coli* are located in this order downstream of the promoter (tetP) of the tetracycline resistance gene of pVIC40 so that the transcription directions of the genes are ordinary with respect to tetP. The mutant *dapA* coded for a mutant DDPS in which the 118-histidine residue was replaced with a thyrosine residue. The mutant *lysC* coded for a mutant AKIII in which the 352-threonine residue was replaced with an isoleucine residue.

**[0093]** RSFD80 was constructed as follows. The mutant *dapA* on a plasmid pdapAS24 was ligated to pVIC40 at a position downstream of the promoter of the tetracycline resistance gene to obtain RSF24P as shown in Fig. 1. Then, the plasmid RSFD80 which had the mutant *dapA* and a mutant *lysC* was prepared from RSF24P and pLLC*80 containing the mutant *lysC* as shown in Fig. 2. That is, while pVIC40 contains a threonine operon, this threonine operon is replaced with a DNA fragment containing the mutant *dapA* and a DNA fragment containing the mutant *lysC* in RSFD80.

**[0094]** The *E. coli* JM109 strain transformed with the RSFD80 plasmid was designated as AJ12396, and deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (postal code 305-8566, 1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on October 28, 1993 and received an accession number of FERM P-13936, and it was transferred to an international deposition under the provisions of the Budapest Treaty on November 1, 1994, and received an accession number of FERM BP-4859.

**[0095]** The *E. coli* AJ1239 strain was cultured in 30 ml of LB medium containing 20 mg/L of streptomycin at 30°C for 12 hours, and the RSFD80 plasmid was purified from the obtained cells by using Wizard® Plus Midipreps DNA Purification System (sold by Promega).

**[0096]** The RSFD80 plasmid produced as described above was introduced into the *Methylophilus methylotrophus* AS1 strain (NCIMB10515) by electroporation (Canadian Journal of Microbiology, 43, 197 (1997)). As a control, a DNA region coding for the threonine operon was deleted from the pVIC40 plasmid used for producing the RSFD80 plasmid to produce a pRS plasmid comprising only the vector region (see Japanese Patent Application Laid-open (Kohyo) No. 3-501682/1991), and the pRS plasmid was introduced into the AS1 strain in the same manner as that used for RSFD80.

(2) AKIII Activity of *Methylophilus* bacterium containing mutant *lysC* and mutant *dapA* derived from *E. coli*

**[0097]** Cell-free extracts were prepared from the *Methylophilus methylotrophus* AS1 strain containing the RSFD80 plasmid (also referred to as "AS1/RSFD80" hereinafter) and the *Methylophilus methylotrophus* AS1 strain containing the pRS plasmid (also referred to as "AS1/pRS" hereinafter), and AK activity was measured. The cell-free extracts (crude enzyme solutions) were prepared as follows. The AS1/RSFD80 strain and AS1/pRS strain were each inoculated to 121 production medium of the above composition containing 20 mg/L of streptomycin, cultured at 37°C for 34 hours with shaking, and then calcium carbonate was removed and cells were harvested.

**[0098]** The bacterial cells obtained as described above were washed with 0.2% KCl under a condition of 0°C, suspended in 20 mM potassium phosphate buffer (pH 7) containing 10 mM $MgSO_4$, 0.8 M $(NH_4)_2SO_4$ and 0.03 M β-mercaptoethanol, and disrupted by sonication (0°C, 200 W, 10 minutes). The sonicated cell suspension was centrifuged at 33,000 rpm for 30 minutes under a condition of 0°C, and the supernatant was separated. To the supernatant, ammonium sulfate was added to 80% saturation, and the mixture was left at 0°C for 1 hour, and centrifuged. The pellet was dissolved in 20 mM potassium phosphate buffer (pH 7) containing 10 mM $MgSO_4$, 0.8 M $(NH_4)_2SO_4$ and 0.03 M β-mercaptoethanol.

**[0099]** The measurement of AK activity was performed in accordance with the method of Stadtman (Stadtman, E. R., Cohen, G.N., LeBras, G., and Robichon-Szulmajster, H., J. Biol. Chem., 236, 2033 (1961)). That is, a reaction solution of the following composition was incubated at 30°C for 45 minutes, and color development was caused by adding a $FeCl_3$ solution (2.8 N HCl: 0.4 ml, 12% TCA: 0.4 ml, 5% $FeCl_3 \cdot 6H_2O$/0.1 N HCl: 0.7 ml). The reaction solution was centrifuged, and absorbance of the supernatant was measured at 540 nm. The activity was represented in terms of the amount of hydroxamic acid produced in 1 minute (1 U = 1 μmol/minute). The molar extinction coefficient was set to be 600. The reaction solution not containing potassium aspartate was used as a blank. When the enzymatic

activity was measured, L-lysine was added to the enzymatic reaction solution at various concentrations to examine degree of inhibition by L-lysine. The results are shown in Table 1.

| (Composition of reaction solution) | |
|---|---|
| Reaction mixture *1 | 0.3 ml |
| Hydroxylamine solution *2 | 0.2 ml |
| 0.1 M Potassium aspartate (pH 7.0) | 0.2 ml |
| Enzyme solution | 0.1 ml |
| Water (balance) | Total 1 ml |

*1: 1 M Tris-HCl (pH 8.1): 9 ml, 0.3 M $MgSO_4$: 0.5 ml and 0.2 M ATP (pH 7.0): 5 ml

*2: 8 M Hydroxylamine solution neutralized with KOH immediately before use

Table 1

| Strain | AK activity (Specific activity*1) | Specific activity with 5 mM L-lysine | Desensitization degree of inhibition*2 (%) |
|---|---|---|---|
| AS1/pRS | 7.93 | 9.07 | 114 |
| AS1/RSFD80 | 13.36 | 15.33 | 115 |

*1: nmol/minute/mg protein

*2: Activity retention ratio in the presence of 5 mM LERR-lysine

**[0100]** As shown in Table 1, AK activity was increased by about 1.7 times by the introduction of the RSFD80 plasmid. Further, it was confirmed that the inhibition by L-lysine was completely desensitized in AK derived from E. *coli* that was encoded by the RSFD80 plasmid. Moreover, it was found that AK that was originally retained by the AS1 strain was not inhibited by L-lysine alone. The inventors of the present invention have discovered that the AK derived from the AS1 strain was inhibited by 100% when 2 mM for each of L-lysine and L-threonine were present in the reaction solution (concerted inhibition).

(3) Production of L-lysine by *Methylophilus* bacterium containing mutant *lysC* and mutant *dapA* derived from *E. coli*

**[0101]** Then, the AS1/RSFD80 strain and the AS1/pRS strain were inoculated to 121 production medium containing 20 mg/L of streptomycin, and cultured at 37°C for 34 hours with shaking. After the culture was completed, the bacterial cells and calcium carbonate were removed by centrifugation, and L-lysine concentration in the culture supernatant was measured by an amino acid analyzer (JASCO Corporation [Nihon Bunko], high performance liquid chromatography). The results are shown in Table 2.

Table 2

| Strain | Production amount of L-lysine hydrochloride (g/L) |
|---|---|
| AS1/pRS | 0 |
| AS1/RSFD80 | 0.3 |

Example 2

Creation of L-lysine-producing bacterium (2)

(1) Introduction of *tac* promoter region into broad host spectrum vector

**[0102]** In order to produce a large amount of enzyme involved in the biosynthesis of L-lysine (Lys) in *Methylophilus methylotrophus*, *tac* promoter was used for gene expression of the target enzyme. The promoter is frequently used in *E. coli*.

**[0103]** The *tac* promoter region was obtained by amplification through PCR using DNA of pKK233-3 (Pharmacia) as a template, DNA fragments having the nucleotide sequences of SEQ ID NOS: 15 and 16 as primers, and a heat-resistant DNA polymerase. The PCR was performed with a cycle of 94°C for 20 seconds, 60°C for 30 seconds, and 72°C for 60 seconds, which was repeated 30 times. Then, the amplified DNA fragment was collected and treated with

restriction enzymes *Eco*RI and *Pst*I. On the other hand, a broad host spectrum vector pRS (see Japanese Patent Application Laid-open (Kohyo) No. 3-501682/1991) was also digested with the same restriction enzymes, and the aforementioned DNA fragment which contained the *tac* promoter region was introduced into the restriction enzyme digestion termini to construct pRS-tac.

(2) Preparation of *dapA* gene (dihydrodipicolinate synthase gene) expression plasmid pRS-dapA24 and *lysC* gene (aspartokinase gene) expression plasmid pRS-lysC80

**[0104]** A mutant gene (*dapA*24*) coding for dihydrodipicolinate synthase whose feedback inhibition for the enzyme activity by Lys was partially desensitized was introduced into the plasmid pRS-tac which was prepared by the method described in the above (1).

**[0105]** First, the *dapA*24* gene region was obtained by amplification through PCR using DNA of RSFD80 (see Example 1) as a template, and DNA fragments having the nucleotide sequences of SEQ ID NOS: 17 and 18 as primers. The PCR was performed with a cycle of 94°C for 20 seconds, 60°C for 30 seconds, and 72°C for 90 seconds, which was repeated 30 times. Then, the fragment was treated with restriction enzymes *Sse*8387I and *Xba*I to prepare a *dapA*24* gene fragment having corresponding cleaved termini. On the other hand, pRS-tac was also treated with *Sse*8387I and partially digested with *Xba*I in the same manner as described above. To this digested plasmid, the aforementioned *dapA*24* gene fragment was ligated by using T4 ligase to obtain pRS-dapA24.

**[0106]** Similarly, a gene (*lysC*80*) coding for aspartokinase whose feedback inhibition for the enzyme activity by Lys was partially desensitized was obtained by PCR using DNA of RSFD80 as a template, and DNA fragments having the nucleotide sequences of SEQ ID NOS: 19 and 20 as primers. The PCR was performed with a cycle of 94°C for 20 seconds, 60°C for 30 seconds, and 72°C for 90 seconds, which was repeated 30 times. Then, the obtained DNA fragment was treated with restriction enzymes *Sse*8387I and *Sap*I. On the other hand, the vector pRS-tac was also treated with *Sse*8387I and *Sap*I. To this digested plasmid, the aforementioned *lysC*80* gene fragment was ligated by using T4 ligase to obtain pRS-lysC80.

(3) Introduction of pRS-dapA24 or pRS-lysC80 into *Methylophilus methylotrophus* and evaluation of culture

**[0107]** Each of pRS-dapA24 and pRS-lysC80 obtained as described above was introduced into the *Methylophilus methylotrophus* AS1 strain (NCIMB10515) by electroporation to obtain AS1/pRS-dapA24 and AS1/pRS-lysC80, respectively. Each strain was inoculated to 121 production medium containing 20 mg/L of streptomycin, and cultured at 37°C for 48 hours with shaking. As a control strain, AS1 strain harboring pRS was also cultured in a similar manner. After the culture was completed, the cells and calcium corbonate were removed by centrifugation, and L-lysine concentration in the culture supernatant was measured by an amino acid analyzer (JASCO Corporation [Nihon Bunko], high performance liquid chromatography). The results are shown in Table 3.

Table 3

| Strain | Production amount of L-lysine hydrochloride (g/L) |
|---|---|
| AS1/pRS | <0.01 |
| AS1/pRS-lysC80 | 0.06 |
| AS1/pRS-dapA24 | 0.13 |

Example 3

Creation of L-lysine-producing bacterium (3)

**[0108]** The *Methylophilus methylotrophus* AS1 strain (NCIMB10515) was inoculated to 121M1 medium and cultured at 37°C for 15 hours. The obtained bacterial cells were treated with NTG in a conventional manner (NTG concentration: 100 mg/L, 37°C, 5 minutes), and spread onto 121M1 agar medium containing 7 g/L of S-(2-aminoethyl)-cysteine (AEC) and 3 g/L of L-threonine. The cells were cultured at 37°C for 2 to 8 days, and the formed colonies were picked up to obtain AEC-resistant strains.

**[0109]** The aforementioned AEC-resistant strains were inoculated to 121 production medium, and cultured at 37°C for 38 hours under an aerobic condition. After the culture was completed, the cells and calcium carbonate were removed from the medium by centrifugation, and L-lysine concentration in the culture supernatant was measured by an amino acid analyzer (JASCO Corporation [Nihon Bunko], high performance liquid chromatography). A strain showing improved L-lysine-producing ability compared with the parent strain was selected, and designated as *Methylophilus meth-*

*ylotrophus* AR-166 strain. The L-lysine production amounts of the parent strain (AS1 strain) and the AR-166 strain are shown in Table 4.

Table 4

| Strain | Production amount of L-lysine hydrochloride (mg/L) |
|---|---|
| AS1 | 5.8 |
| AR-166 | 80 |

**[0110]** The *Methylophilus methylotrophus* AR-166 strain was given a private number of AJ13608, and was deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (postal code 305-8566, 1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on June 10, 1999 and received an accession number of FERM P-17416, and it was transferred to an international deposition under the provisions of the Budapest Treaty on March 31, 2000, and received an accession number of FERM BP-7112.

Example 4

Creation of L-threonine-producing bacterium

(1) Introduction of threonine operon plasmid into *Methylophilus* bacterium

**[0111]** A plasmid pVIC40 (International Publication WO90/04636, Japanese Patent Application Laid-open (Kohyo) No. 3-501682/1991) containing a threonine operon derived from *E. coli* was introduced into the *Methylophilus methylotrophus* AS1 strain (NCIMB10515) by electroporation (Canadian Journal of Microbiology, 43, 197 (1997)) to obtain AS1/pVIC40 strain. As a control, pRS (Japanese Patent Application Laid-open (Kohyo) No. 3-501682/1991) having only the vector region was obtained by deleting the DNA region coding for the threonine operon from the pVIC40 plasmid, and it was introduced into the AS1 strain in the same manner as used for pVIC40 to obtain AS1/pRS strain.

(2) Production of L-threonine by *Methylophilus* bacterium containing threonine operon derived from *E. coli*

**[0112]** Each of the AS1/pVIC40 and AS1/pRS strains was inoculated to 121 production medium containing 20 mg/L of streptomycin, 1 g/l of L-valine and 1 g/l of L-leucine, and cultured at 37°C for 50 hours with shaking. After the culture was completed, the cells and calcium carbonate were removed by centrifugation, and L-threonine concentration in the culture supernatant was measured by an amino acid analyzer (JASCO Corporation [Nihon Bunko], high performance liquid chromatography). The results are shown in Table 5.

Table 5

| Strain | Production amount of L-threonine (mg/L) |
|---|---|
| AS1/pRS | 15 |
| AS1/pVIC40 | 30 |

Example 5

Creation of branched chain amino acid-producing bacterium

**[0113]** The *Methylophilus methylotrophus* AS1 strain (NCIMB10515) was inoculated to 121M1 medium and cultured at 37°C for 15 hours. The obtained bacterial cells were treated with NTG in a conventional manner (NTG concentration: 100 mg/L, 37°C, 5 minutes), and spread onto 121M1 agar medium containing 0.5% of casamino acid (DIFCO). The cells were cultured at 37°C for 2 to 8 days, and allowed to form colonies. The formed colonies were picked up, and inoculated to 121M1 agar medium and 121M1 agar medium containing 0.5% of casamino acid. Strains exhibiting better growth on the latter medium compared with on the former medium were selected as casamino acid auxotrophic strains. In this way, 9 leaky casamino acid auxotrophic strains were obtained from NTG-treated 500 strains. From these casamino acid auxotrophic strains, one strain that accumulated more L-valine, L-leucine and L-isoleucine in the medium compared with its parent strain was obtained. This strain was designated as *Methylophilus methylotrophus* C138 strain.

**[0114]** The *Methylophilus methylotrophus* C138 strain was given a private number of AJ13609, and was deposited

at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (postal code 305-8566, 1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on June 10, 1999 and received an accession number of FERM P-17417, and it was transferred to an international deposition under the provisions of the Budapest Treaty on March 31, 2000, and received an accession number of FERM BP-7113.

**[0115]** The parent strain (AS1 strain) and the C138 strain were inoculated to 121 production medium, and cultured at 37°C for 34 hours under an aerobic condition. After the culture was completed, the cells and calcium carbonate were removed from the medium by centrifugation, and concentrations of L-valine, L-leucine and L-isoleucine in the culture supernatant were measured by an amino acid analyzer (JASCO Corporation [Nihon Bunko], high performance liquid chromatography). The results are shown in Table 6.

Table 6

| Strain | L-valine (mg/L) | L-leucine (mg/L) | L-isoleucine (mg/L) |
|---|---|---|---|
| AS1 | 7.5 | 5.0 | 2.7 |
| C138 | 330 | 166 | 249 |

Example 6

Preparation of chromosome DNA library of *Methylophilus methylotrophus* AS1 strain

(1) Preparation of chromosome DNA of *Methylophilus methylotrophus* AS1 strain

**[0116]** One platinum loop of the *Methylophilus methylotrophus* AS1 strain (NCIMB10515) was inoculated to 5 ml of 121M1 medium in a test tube, and cultured at 37°C overnight with shaking. The obtained culture broth was inoculated to 50 ml of 121M1 medium in a 500 ml-volume Sakaguchi flask in an amount of 1%, and cultured at 37°C overnight with shaking. Then, the cells were harvested by centrifugation, and suspended in 50 ml of TEN solution (solution containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA and 20 mM NaCl (pH 8.0)). The cells were collected by centrifugation, and suspended again in 5 ml of the TEN solution containing 5 mg/ml of lysozyme and 10 μg/ml of RNase A. The suspension was maintained at 37°C for 30 minutes, and then proteinase K and sodium laurylsulfate were added thereto to final concentrations of 10 μg/ml and 0.5% (wt/vol), respectively.

**[0117]** The suspension was maintained at 70°C for 2 hours, and then an equal amount of a saturated solution of phenol (phenol solution saturated with 10 mM Tris-HCl (pH 8.0)) was added and mixed. The suspension was centrifuged, and the supernatant was collected. An equal amount of phenol/chloroform solution (phenol:chloroform:isoamyl alcohol = 25:24:1) was added and mixed, and the mixture was centrifuged. The supernatant was collected, and an equal amount of chloroform solution (chloroform:isoamyl alcohol = 24:1) was added thereto to repeat the same extraction procedure. To the supernatant, a 1/10 volume of 3 M sodium acetate (pH 4.8) and 2.5-fold volume of ethanol were added to precipitate chromosome DNA. The precipitates were collected by centrifugation, washed with 70% ethanol, dried under reduced pressure, and dissolved in a suitable amount of TE solution (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)).

(2) Preparation of gene library

**[0118]** A 50 μl portion of the chromosome DNA (1 μg/μl) obtained in the above (1), 20 μl of H buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM dithiothreitol, 1000 mM NaCl (pH 7.5)) and 8 units of a restriction enzyme *Sau*3AI (Takara Shuzo) were allowed to react at 37°C for 10 minutes in a total volume of 200 μl, and then 200 μl of the phenol/chloroform solution was added and mixed to stop the reaction. The reaction mixture was centrifuged, and the upper layer was collected and separated on a 0.8% agarose gel. DNA corresponding to 2 to 5 kilobase pair (henceforth abbreviated as "kbp") was collected by using Concert™ Rapid Gel Extraction System (DNA collecting kit, GIBCO BRL Co.). In this way, 50 μl of a solution of DNA with fractionated size was obtained.

**[0119]** On the other hand, 2.5 μg of plasmid pUC118 (Takara Shuzo), 2 μl of K buffer (200 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM dithiothreitol, 1000 mM KCl (pH 8.5)) and 10 units of restriction enzyme *Bam*HI (Takara Shuzo) were allowed to react at 37°C for 2 hours in a total volume of 20 μl, then 20 units of calf small intestine alkaline phosphatase (Takara Shuzo) was added and mixed, and the mixture was allowed to react for further 30 minutes. The reaction mixture was mixed with an equal amount of the phenol/chloroform solution, and the mixture was centrifuged. The supernatant was collected, and an equal amount of the chloroform solution was added thereto to repeat a similar extraction procedure. To the supernatant, a 1/10 volume of 3 M sodium acetate (pH 4.8) and 2.5-fold volume of ethanol were added to precipitate DNA. The DNA was collected by centrifugation, washed with 70% ethanol, dried under reduced pressure,

and dissolved in a suitable amount of TE solution.

**[0120]** A *Sau*3AI digestion product of the chromosome DNA prepared as described above and a *Bam*HI digestion product of pUC118 were ligated by using a Ligation Kit ver. 2 (Takara Shuzo). To the reaction mixture, a 1/10 volume of 3 M sodium acetate (pH 4.8) and 2.5-fold volume of ethanol were added to precipitate DNA. The DNA was collected by centrifugation, washed with 70% ethanol, dried under reduced pressure, and dissolved in TE solution (Ligase solution A).

**[0121]** In the same manner as in the above procedure, fragments obtained by partial digestion of the chromosome DNA with a restriction enzyme *Alu*I (Takara Shuzo) and a *Sma*I digestion product of plasmid pSTV29 (Takara Shuzo) were ligated (Ligase solution B).

**[0122]** One platinum loop of *E. coli* JM109 was inoculated to 5 ml of L medium in a test tube, and cultured at 37°C overnight with shaking. The obtained culture broth was inoculated to 50 ml of L medium in a 500 ml-volume Sakaguchi flask in an amount of 1%, cultured at 37°C until $OD_{660}$ of the culture became 0.5 to 0.6, and cooled on ice for 15 minutes. Then, the cells were harvested by centrifugation at 4°C. The cells were suspended in 50 ml of ice-cooled water and centrifuged to wash the cells. This operation was repeated once again, and the cells were suspended in 50 ml of ice-cooled 10% glycerol solution, and centrifuged to wash the cells. The cells were suspended in 10% glycerol solution of the same volume as the cells, and divided into 50 µl aliquots. To the cells in the 50 µl volume, 1 µl of Ligase solution A or Ligase solution B prepared above was added. Then, the mixture was put into a special cuvette (0.1 cm width, preliminarily ice-cooled) for an electroporation apparatus of BioRad.

**[0123]** The setting of the apparatus was 1.8 kV and 25 µF, and the setting of pulse controller was 200 ohms. The cuvette was mounted on the apparatus and pulses were applied thereto. Immediately after the application of pulse, 1 ml of ice-cooled SOC medium was added thereto, and the mixture was transferred into a sterilized test tube, and cultured at 37°C for 1 hour with shaking. Each cell culture broth was spread onto L agar medium containing an antibiotic (100 µg/ml of ampicillin when Ligase solution A was used, or 20 µg/ml of chloramphenicol when Ligase solution B was used), and incubated at 37°C overnight. The colonies emerged on each agar medium were scraped, inoculated to 50 ml of L medium containing respective antibiotic in a 500 ml-volume Sakaguchi flask, and cultured at 37°C for 2 hours with shaking. Plasmid DNA was extracted from each culture broth by the alkali SDS method to form Gene library solution A and Gene library solution B, respectively.

Example 7

Cloning of lysine biosynthesis gene of *Methylophilus methylotrophus* AS1 strain

(1) Cloning of gene coding for aspartokinase (AK)

**[0124]** *E. coli* GT3 deficient in the three genes coding for AK (*thrA, metLM* and *lysC)* was transformed with Gene library solution B by the same electroporation procedure as mentioned above. SOC medium containing 20 µg/ml of diaminopimelic acid was added to the transformation solution, and cultured at 37°C with shaking. Then, the culture broth was spread onto L medium containing 20 µg/ml of diaminopimelic acid and 20 µg/ml of chloramphenicol to obtain emerged colonies. This was replicated as a master plate to M9 agar medium containing 20 µg/ml of chloramphenicol, and the replicate was incubated at 37°C for 2 to 3 days. The host could not grow in M9 minimal medium that did not contain diaminopimelic acid since it did not have AK activity. In contrast, it was expected that the transformant strain that contained the gene coding for AK derived from *Methylophilus methylotrophus* could grow in M9 minimal medium because of the function of the gene

**[0125]** Two transformants out of about 3000 transformants formed colonies on M9 medium. Plasmids were extracted from the colonies emerged on M9 medium and analyzed. As a result, the presence of an inserted fragment on the plasmids was confirmed. The plasmids were designated as pMMASK-1 and pMMASK-2, respectively. By using these plasmids, *E. coli* GT3 was transformed again. The obtained transformants could grow on M9 minimal medium. Further, the transformant which contained each of these plasmids was cultured overnight in L medium containing 20 µg/ml of chloramphenicol, and the cells were collected by centrifugation of the culture broth. Cell-free extracts were prepared by sonicating the cells, and AK activity was measured according to the method of Miyajima et al. (Journal of Biochemistry (Tokyo), vol. 63, 139-148 (1968)) (Fig. 3: pMMASK-1, pMMASK-2). In addition, a GT3 strain harboring the vector pSTV29 was similarly cultured in L medium containing 20 µg/ml of diaminopimelic acid and 20 µg/ml of chloramphenicol, and AK activity was measured (Fig. 3: Vector). As a result, increase in AK activity was observed in two of the clones containing the inserted fragments compared with the transformant harboring only the vector. Therefore, it was confirmed that the gene that could be cloned on pSTV29 was a gene coding for AK derived from *Methylophilus methylotrophus*. This gene was designated as *ask*.

**[0126]** The DNA nucleotide sequence of the *ask* gene was determined by the dideoxy method. It was found that pMMASK-1 and pMMASK-2 contained a common fragment. The nucleotide sequence of the DNA fragment containing

the *ask* gene derived from *Methylophilus methylotrophus* is shown in SEQ ID NO: 5. An amino acid sequence that can be encoded by the nucleotide sequence is shown in SEQ ID NOS: 5 and 6.

(2) Cloning of gene coding for aspartic acid semialdehyde dehydrogenase (ASD)

**[0127]** *E. coli* Hfr3000 U482 (CGSC 5081 strain) deficient in the *asd* gene was transformed by electroporation using Gene library solution B in the same manner as described above. To the transformation solution, SOC medium containing 20 μg/ml of diaminopimelic acid was added and the mixture was cultured at 37°C with shaking. The cells were harvested by centrifugation. The cells were washed by suspending them in L medium and centrifuging the suspension. The same washing operation was repeated once again, and the cells were suspended in L medium. Then, the suspension was spread onto L agar medium containing 20 μg/ml of chloramphenicol, and incubated overnight at 37°C. The host showed extremely slow growth in L medium not containing diaminopimelic acid since it was deficient in the asd gene. In contrast, it was expected that normal growth would be observed for a transformant strain which contained the gene coding for ASD derived from *Methylophilus methylotrophus* even in L medium because of the function of the gene. Further, the host *E. coli* could not grow in M9 minimal medium, but a transformant strain that contained the gene coding for ASD derived from *Methylophilus methylotrophus* was expected to be able to grow in M9 minimal medium because of the function of the gene. Therefore, colonies of transformants that normally grew on L medium were picked up, streaked and cultured on M9 agar medium. As a result, growth was observed. Thus, it was confirmed that the gene coding for ASD functioned in these transformant strains as expected.

**[0128]** Plasmids were extracted from the three transformant strains emerged on M9 medium, and the presence of an inserted fragment in the plasmids was confirmed. The plasmids were designated as pMMASD-1, pMMASD-2 and pMMASD-3, respectively. When the *E. coli* Hfr3000 U482 was transformed again by using these plasmids, each transformant grew in M9 minimal medium. Further, each transformant was cultured overnight in L medium containing 20 μg/ml of chloramphenicol, and the cells were collected by centrifugation of the culture broth. The cells were sonicated to prepare a crude enzyme solution, and ASD activity was measured according to the method of Boy et al. (Journal of Bacteriology, vol. 112 (1), 84-92 (1972)) (Fig. 4: pMMASD-1, pMMASD-2, pMMASD-3). In addition, the host harboring the vector was similarly cultured in L medium containing 20 μg/ml of diaminopimelic acid and 20 μg/ml of chloramphenicol, and ASD activity was measured as a control experiment (Fig. 4: Vector). As a result, the enzymatic activity could not be detected for the transformant harboring only the vector, whereas the ASD activity could be detected in three of the clones having an insert fragment. Therefore, it was confirmed that the obtained gene was a gene coding for ASD derived from *Methylophilus methylotrophus* (designated as *asd*).

**[0129]** The DNA nucleotide sequence of the *asd* gene was determined by the dideoxy method. It was found that all of the three obtained clones contained a common fragment. The nucleotide sequence of the DNA fragment containing the *asd* gene derived from *Methylophilus methylotrophus* is shown in SEQ ID NO: 7. An amino acid sequence that can be encoded by the nucleotide sequence is shown in SEQ ID NOS: 7 and 8.

(3) Cloning of gene coding for dihydrodipicolinate synthase (DDPS)

**[0130]** *E. coli* AT997 (CGSC 4547 strain) deficient in the *dapA* gene was transformed by the same electroporation procedure using Gene library solution A. To the transformation solution, SOC medium containing 20 μg/ml of diaminopimelic acid was added, and the mixture was cultured at 37°C with shaking. Then, the culture broth was spread onto L medium containing 20 μg/ml of diaminopimelic acid and 100 μg/ml of ampicillin to obtain emerged colonies. This was replicated as a master plate to M9 minimal agar medium containing 100 μg/ml of ampicillin, and the replicate was incubated at 37°C for 2 to 3 days. The host could not grow in M9 minimal medium that did not contain diaminopimelic acid since it was deficient in *dapA* gene. In contrast, it was expected that a transformant strain that contained the gene coding for DDPS derived from *Methylophilus methylotrophus* could grow in M9 minimal medium because of the function of that gene.

**[0131]** Plasmids were extracted from the colonies of two strains emerged on M9 medium, and analyzed. As a result, the presence of an inserted fragment in the plasmids was confirmed. The plasmids were designated as pMMDAPA-1 and pMMDAP-2, respectively. When *E. coli* AT997 was transformed again by using these plasmids, each transformant was grown in M9 minimal medium. Further, each transformant containing each plasmid was cultured overnight in L medium containing 100 μg/ml of ampicillin, and the cells were collected by centrifugation of the culture broth. The cells were sonicated to prepare a cell extract, and DDPS activity was measured according to the method of Yugari et al. (Journal of Biological Chemistry, vol.240, and p.4710 (1965)) (Fig. 5: pMMDAPA-1, pMMDAPA-2). In addition, the host harboring the vector was similarly cultured in L medium containing 20 μg/ml of diaminopimelic acid and 100 μg/ml of ampicillin, and DDPS activity was measured as a control experiment (Fig. 5: Vector). As a result, the enzymatic activity could not be detected for the transformant harboring only the vector, whereas the DDPS activity could be detected in each of the transformants harboring the plasmids having the insert fragment. Therefore, it was confirmed that the

obtained gene was a gene coding for DDPS derived from *Methylophilus methylotrophus* (designated as *dapA*).

**[0132]** The DNA nucleotide sequence of the *dapA* gene was determined by the dideoxy method. It was found that two of the inserted fragments contained a common fragment. The nucleotide sequence of the DNA fragment containing the *dapA* gene derived from *Methylophilus methylotrophus* is shown in SEQ ID NO: 9. An amino acid sequence that can be encoded by the nucleotide sequence is shown in SEQ ID NOS: 9 and 10.

(4) Cloning of gene coding for dihydrodipicolinate reductase (DDPR)

**[0133]** *E. coli* AT999 (CGSC 4549 strain) deficient in the *dapB* gene was transformed by the same electroporation procedure as described above using Gene library solution A. To the transformation solution, SOC medium containing 20 μg/ml of diaminopimelic acid was added, and the mixture was cultured at 37°C with shaking. Then, the cells were harvested by centrifugation. The cells were washed by suspending them in L medium and centrifuging the suspension. The same washing operation was repeated once again, and the cells were suspended in L medium. Then, the suspension was spread onto L agar medium containing 100 μg/ml of ampicillin, and incubated overnight at 37°C. The host showed extremely slow growth in L medium not containing diaminopimelic acid since it was deficient in the *dapB* gene. In contrast, it was expected that normal growth could be observed for a transformant strain that contained the gene coding for DDPR derived from *Methylophilus methylotrophus* even in L medium because of the function of the gene. Further, the host *E. coli* could not grow in M9 minimal medium, but it was expected that a transformant strain which contained the gene coding for DDPR derived from *Methylophilus methylotrophus* could grow in M9 minimal medium because of the function of the gene.

**[0134]** Therefore, a colony of transformant that normally grew on L medium was streaked and cultured on M9 agar medium. Then, growth was also observed on M9 medium. Thus, it was confirmed that the gene coding for DDPR functioned in the transformant strain. A plasmid was extracted from the colony emerged on M9 medium, and the presence of an inserted fragment in the plasmid was confirmed. When *E. coli* AT999 was transformed again by using the plasmid (pMMDAPB), the transformant grew in M9 minimal medium. Further, the transformant containing the plasmid was cultured overnight in L medium, and the cells were collected by centrifugation of the culture broth. The cells were sonicated to prepare a cell extract, and DDPR activity was measured according to the method of Tamir et al. (Journal of Biological Chemistry, vol. 249, p.3034 (1974)) (Fig. 6: pMMDAPB). In addition, the host harboring the vector was similarly cultured in L medium containing 20 μg/ml diaminopimelic acid and 100 μg/ml of ampicillin, and DDPR activity was measured as a control experiment (Fig. 6: Vector). As a result, the enzymatic activity could not be detected for the transformant harboring only the vector, whereas the DDPR activity could be detected for the transformant harboring pMMDAPB. Therefore, it was confirmed that the obtained gene was a gene coding for DDPR derived from *Methylophilus methylotrophus* (designated as *dapB)*.

**[0135]** The DNA nucleotide sequence of the *dapB* gene was determined by the dideoxy method. The nucleotide sequence of the DNA fragment containing the *dapB* gene derived from *Methylophilus methylotrophus* is shown in SEQ ID NO: 11. An amino acid sequence that can be encoded by the nucleotide sequence is shown in SEQ ID NOS: 11 and 12.

(5) Cloning of gene coding for diaminopimelate decarboxylase (DPDC)

**[0136]** *E. coli* AT2453 (CGSC 4505 strain) deficient in the *lysA* gene was transformed by the same electroporation procedure as described above using Gene library solution A. The transformation solution, SOC medium was added, and the mixture was cultured at 37°C with shaking. The cells were harvested by centrifugation. The cells were washed by suspending them in 5 ml of sterilized water and centrifuging the suspension. The same washing operation was repeated once again, and the cells were suspended in 500 μl of sterilized water. Then, the suspension was spread onto M9 minimal agar medium containing 20 μg/ml of chloramphenicol, and incubated at 37°C for 2 to 3 days. The host could not grow in M9 minimal medium not containing lysine since it was deficient in the *lysA* gene. In contrast, it was expected that a transformant strain that contained the gene coding for DPDC derived from *Methylophilus methylotrophus* could grow in M9 minimal medium because of the function of the gene.

**[0137]** Therefore, plasmids were extracted from the three transformant strains emerged on M9 medium, and analyzed. As a result, the presence of an inserted fragment in the plasmids was confirmed. The plasmids were designated as pMMLYSA-1, pMMLYSA-2 and pMMLYSA-3, respectively. When *E. coli* AT2453 was transformed again by using each of these plasmids, each transformant grew in M9 minimal medium. Further, each transformant containing each plasmid was cultured overnight in L medium containing 20 μg/ml of chloramphenicol, and the cells were collected by centrifugation of the culture broth. The cells were sonicated to prepare a cell extract, and DPDC activity was measured according to the method of Cremer et al. (Journal of General Microbiology, vol. 134, 3221-3229 (1988)) (Fig. 7: pMMLYSA-1, pMMLYSA-2, pMMLYSA-3). In addition, the host harboring the vector was similarly cultured in L medium containing 20 μg/ml of chloramphenicol, and DPDC activity was measured as a control experiment (Fig. 7: Vector). As a

result, the enzymatic activity could not be detected for the transformant harboring only the vector, whereas the DPDC activity could be detected in three of the clones having an insert fragment. Therefore, it was confirmed that the obtained gene was a gene coding for DPDC derived from *Methylophilus methylotrophus* (designated as *lysA*).

**[0138]** The DNA nucleotide sequence of the *lysA* gene was determined by the dideoxy method. It was found that all of the three inserted fragments contained a common DNA fragment. The nucleotide sequence of the DNA fragment containing the *lysA* gene derived from *Methylophilus methylotrophus* is shown in SEQ ID NO: 13. An amino acid sequence that can be encoded by the nucleotide sequence is shown in SEQ ID NOS: 13 and 14.

Industrial Applicability

**[0139]** According to the present invention, there are provided a *Methylophilus* bacterium having L-amino acid-producing ability, a method for producing an L-amino acid using the *Methylophilus* bacterium, and *Methylophilus* bacterial cells with increased content of an L-amino acid. By the method of the present invention, it is enabled to produce an L-amino acid using methanol as a raw material. Moreover, novel L-lysine biosynthesis enzyme genes derived from *Methylophilus* bacteria are provided by the present invention.

# SEQUENCE LISTING

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tca gat act act ggt gta gca ctg gct gcg gcg tta aag gcg gat gaa   1013
Ser Asp Thr Thr Gly Val Ala Leu Ala Ala Ala Leu Lys Ala Asp Glu
        155                 160                 165
tgt cag att tat acc gat gtc gat ggc gtt tac acc acc gat ccg cgt   1061
Cys Gln Ile Tyr Thr Asp Val Asp Gly Val Tyr Thr Thr Asp Pro Arg
    170                 175                 180
gtg gtg cct gag gca cgc cgc ttg gat aaa att acc ttt gaa gaa atg   1109
Val Val Pro Glu Ala Arg Arg Leu Asp Lys Ile Thr Phe Glu Glu Met
185                 190                 195                 200
ttg gaa ctg gct tca cag ggc tcc aaa gta ttg caa att cgc tcg gtt   1157
Leu Glu Leu Ala Ser Gln Gly Ser Lys Val Leu Gln Ile Arg Ser Val
                205                 210                 215
gag ttt gcc ggt aaa tac aaa gtc aaa tta cgt gtg ctg tcc agc ttc   1205
Glu Phe Ala Gly Lys Tyr Lys Val Lys Leu Arg Val Leu Ser Ser Phe
            220                 225                 230
gaa gag gag ggc gac ggt aca ctg atc aca ttc gaa gaa aat gag gaa   1253
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```
Glu Glu Glu Gly Asp Gly Thr Leu Ile Thr Phe Glu Glu Asn Glu Glu
        235                 240                 245
aac atg gaa gaa cca att atc tcc ggc atc gcc ttt aac cgc gat gag   1301
Asn Met Glu Glu Pro Ile Ile Ser Gly Ile Ala Phe Asn Arg Asp Glu
    250                 255                 260
gcg aaa att acc gtg acg ggc gtg ccc gac aaa cca gga att gcc tat   1349
Ala Lys Ile Thr Val Thr Gly Val Pro Asp Lys Pro Gly Ile Ala Tyr
265                 270                 275                 280
cag att ttg ggc ccg gtg gca gac gcc aat att gat gtg gat atg att   1397
Gln Ile Leu Gly Pro Val Ala Asp Ala Asn Ile Asp Val Asp Met Ile
                285                 290                 295
atc cag aac gtc ggt gcg gat ggt acg act gac ttc acc ttt acc gta   1445
Ile Gln Asn Val Gly Ala Asp Gly Thr Thr Asp Phe Thr Phe Thr Val
            300                 305                 310
cat aaa aat gag atg aac aaa gcc ctg agc att ctt aga gat aaa gtg   1493
His Lys Asn Glu Met Asn Lys Ala Leu Ser Ile Leu Arg Asp Lys Val
        315                 320                 325
cag ggc cat atc cag gca cgt gaa atc agc ggc gac gac aag att gcc   1541
Gln Gly His Ile Gln Ala Arg Glu Ile Ser Gly Asp Asp Lys Ile Ala
    330                 335                 340
aaa gtc tct gtg gtt ggg gtg ggt atg cgc tca cat gta ggg atc gcc   1589
Lys Val Ser Val Val Gly Val Gly Met Arg Ser His Val Gly Ile Ala
345                 350                 355                 360
agc cag atg ttc cgt acg ctg gcc gaa gaa ggg atc aat att caa atg   1637
Ser Gln Met Phe Arg Thr Leu Ala Glu Glu Gly Ile Asn Ile Gln Met
                365                 370                 375
atc tca acc agc gaa att aaa att gca gtc gtg atc gaa gag aag tac   1685
Ile Ser Thr Ser Glu Ile Lys Ile Ala Val Val Ile Glu Glu Lys Tyr
            380                 385                 390
atg gaa ctg gct gta cgc gtg ttg cat aaa gca ttc ggc ctc gaa aac   1733
Met Glu Leu Ala Val Arg Val Leu His Lys Ala Phe Gly Leu Glu Asn
        395                 400                 405
gca taatcgccaa cggacgaata aagaaataaa acattcttct tttttgcgtt        1786
Ala
gatttttgaa gggttttcac gtagtatggc agcccttcga tgcagtagca atgctgcaaa 1846
gagaacagca tgccgctgtg ttggtactat taaaacttca ttgttttaat aaggtgaggg 1906
ggatcctcta gagtcgacct gcaggcatgc aagcttggcc gtaatccatg gtcatagctg 1966
tttcctggtg tgaaa                                                 1981
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<210> 6
<211> 409
<212> PRT
<213> Methylophilus methylotrophus

<400> 6

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Met Ala Leu Ile Val Gln Lys Tyr Gly Gly Thr Ser Val Ala Asn Pro
 1               5                  10                  15
Glu Arg Ile Arg Asn Val Ala Arg Arg Val Ala Arg Tyr Lys Ala Leu
            20                  25                  30
Gly His Gln Val Val Val Val Val Ser Ala Met Ser Gly Glu Thr Asn
        35                  40                  45
Arg Leu Ile Ser Leu Ala Lys Glu Ile Met Gln Asp Pro Asp Pro Arg
    50                  55                  60
Glu Leu Asp Val Met Val Ser Thr Gly Glu Gln Val Thr Ile Gly Met
65                  70                  75                  80
Thr Ala Leu Ala Leu Met Glu Leu Gly Ile Lys Ala Lys Ser Tyr Thr
                85                  90                  95
Gly Thr Gln Val Lys Ile Leu Thr Asp Asp Ala Phe Thr Lys Ala Arg
            100                 105                 110
Ile Leu Asp Ile Asp Glu His Asn Leu Lys Lys Asp Leu Asp Asp Gly
        115                 120                 125
Tyr Val Cys Val Val Ala Gly Phe Gln Gly Val Asp Ala Asn Gly Asn
    130                 135                 140
Ile Thr Thr Leu Gly Arg Gly Gly Ser Asp Thr Thr Gly Val Ala Leu
145                 150                 155                 160
Ala Ala Ala Leu Lys Ala Asp Glu Cys Gln Ile Tyr Thr Asp Val Asp
                165                 170                 175
Gly Val Tyr Thr Thr Asp Pro Arg Val Val Pro Glu Ala Arg Arg Leu
            180                 185                 190
Asp Lys Ile Thr Phe Glu Glu Met Leu Glu Leu Ala Ser Gln Gly Ser
        195                 200                 205
Lys Val Leu Gln Ile Arg Ser Val Glu Phe Ala Gly Lys Tyr Lys Val
    210                 215                 220
Lys Leu Arg Val Leu Ser Ser Phe Glu Glu Glu Gly Asp Gly Thr Leu
225                 230                 235                 240
Ile Thr Phe Glu Glu Asn Glu Glu Asn Met Glu Glu Pro Ile Ile Ser
```

```
                245                 250                 255
Gly Ile Ala Phe Asn Arg Asp Glu Ala Lys Ile Thr Val Thr Gly Val
            260                 265                 270
Pro Asp Lys Pro Gly Ile Ala Tyr Gln Ile Leu Gly Pro Val Ala Asp
        275                 280                 285
Ala Asn Ile Asp Val Asp Met Ile Ile Gln Asn Val Gly Ala Asp Gly
    290                 295                 300
Thr Thr Asp Phe Thr Phe Thr Val His Lys Asn Glu Met Asn Lys Ala
305                 310                 315                 320
Leu Ser Ile Leu Arg Asp Lys Val Gln Gly His Ile Gln Ala Arg Glu
                325                 330                 335
Ile Ser Gly Asp Asp Lys Ile Ala Lys Val Ser Val Val Gly Val Gly
            340                 345                 350
Met Arg Ser His Val Gly Ile Ala Ser Gln Met Phe Arg Thr Leu Ala
        355                 360                 365
Glu Glu Gly Ile Asn Ile Gln Met Ile Ser Thr Ser Glu Ile Lys Ile
    370                 375                 380
Ala Val Val Ile Glu Glu Lys Tyr Met Glu Leu Ala Val Arg Val Leu
385                 390                 395                 400
His Lys Ala Phe Gly Leu Glu Asn Ala
                405
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<210> 7
<211> 1452
<212> DNA
<213> Methylophilus methylotrophus

<220>
<221> CDS
<222> (98)..(1207)

<400> 7

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gcatgcccgc aggtcgactc tagaggatcc ccctgttcaa aaatcttcca aataatcact 60
gtaatgccgg gttgtccggc tgaaatatcg agtcact atg tta aaa gta ggg ttt  115
                                        Met Leu Lys Val Gly Phe
                                          1               5
gta ggc tgg cgt ggc atg gtt gga tcc gtg cta atg cag cgc atg atg   163
Val Gly Trp Arg Gly Met Val Gly Ser Val Leu Met Gln Arg Met Met
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```
                10                  15                  20
cag gaa aac gat ttt gcg gat att gaa ccg caa ttc ttt acg acc tca   211
Gln Glu Asn Asp Phe Ala Asp Ile Glu Pro Gln Phe Phe Thr Thr Ser
        25                  30                  35
caa acg gga ggg gct gcg cct aaa gtt gga aaa gat act cct gcg ctg   259
Gln Thr Gly Gly Ala Ala Pro Lys Val Gly Lys Asp Thr Pro Ala Leu
    40                  45                  50
aaa gat gcc aag gat att gat gct ttg cgc cag atg gat gtg att gtg   307
Lys Asp Ala Lys Asp Ile Asp Ala Leu Arg Gln Met Asp Val Ile Val
55                  60                  65                  70
acc tgc cag ggt ggc gat tac acg agt gac gtc ttc cca caa ttg cgc   355
Thr Cys Gln Gly Gly Asp Tyr Thr Ser Asp Val Phe Pro Gln Leu Arg
                75                  80                  85
gca acc ggc tgg agc ggc cac tgg att gac gcg gcc tct acc tta cgc   403
Ala Thr Gly Trp Ser Gly His Trp Ile Asp Ala Ala Ser Thr Leu Arg
            90                  95                  100
atg gaa aaa gac tcc gtg atc att tta gac ccg gtg aac atg cat gtg   451
Met Glu Lys Asp Ser Val Ile Ile Leu Asp Pro Val Asn Met His Val
        105                 110                 115
att aaa gat gca ttg tcc aat ggc ggc aaa aac tgg atc ggc ggc aac   499
Ile Lys Asp Ala Leu Ser Asn Gly Gly Lys Asn Trp Ile Gly Gly Asn
    120                 125                 130
tgt acc gtc tca ctt atg ttg atg gcg ctg aat ggc ctg ttt aag gct   547
Cys Thr Val Ser Leu Met Leu Met Ala Leu Asn Gly Leu Phe Lys Ala
135                 140                 145                 150
gac ctg gtc gag tgg gcc act tcc atg acc tac cag gcg gct tca ggc   595
Asp Leu Val Glu Trp Ala Thr Ser Met Thr Tyr Gln Ala Ala Ser Gly
                155                 160                 165
gca ggc gcg cag aat atg cgt gaa ctg att agc cag atg ggc gta gtg   643
Ala Gly Ala Gln Asn Met Arg Glu Leu Ile Ser Gln Met Gly Val Val
            170                 175                 180
aat gcc tcc gtg gct gat ttg ctg gcg gat cca gct tct gcc att ttg   691
Asn Ala Ser Val Ala Asp Leu Leu Ala Asp Pro Ala Ser Ala Ile Leu
        185                 190                 195
cag atc gat aaa aca gtg gcg gat acc atc cgt agc gaa gag ttg cct   739
Gln Ile Asp Lys Thr Val Ala Asp Thr Ile Arg Ser Glu Glu Leu Pro
    200                 205                 210
aaa tct aac ttt ggt gtg cca ttg gcg ggc agt ctg atc cca tgg atc   787
```

```
Lys Ser Asn Phe Gly Val Pro Leu Ala Gly Ser Leu Ile Pro Trp Ile
215                 220                 225                 230
gac aag gac tta ggg aat ggt caa agt aaa gaa gaa tgg aag ggc ggc   835
Asp Lys Asp Leu Gly Asn Gly Gln Ser Lys Glu Glu Trp Lys Gly Gly
                235                 240                 245
gta nag acc aat aag att tta ggt cgt gaa gcg aac ccg att gtg att   883
Val Xaa Thr Asn Lys Ile Leu Gly Arg Glu Ala Asn Pro Ile Val Ile
            250                 255                 260
gac ggt ttg tgt gta cgt atc ggc gcc atg cgt tgc cat tca caa gcg   931
Asp Gly Leu Cys Val Arg Ile Gly Ala Met Arg Cys His Ser Gln Ala
        265                 270                 275
ttg act atc aag ctg cgc aag gat gtg ccg ctg gat gaa atc aat cag   979
Leu Thr Ile Lys Leu Arg Lys Asp Val Pro Leu Asp Glu Ile Asn Gln
    280                 285                 290
atg ctg gct gaa gcg aac gac tgg gct aaa gtc att ccc aat gag cgt   1027
Met Leu Ala Glu Ala Asn Asp Trp Ala Lys Val Ile Pro Asn Glu Arg
295                 300                 305                 310
gag gtc agt atg cgg gaa ctc acc ccg gca gcg att acc ggc agt ctg   1075
Glu Val Ser Met Arg Glu Leu Thr Pro Ala Ala Ile Thr Gly Ser Leu
                315                 320                 325
gcg acg cca gta ggg cgt ttg cgc aaa ctg gcg atg ggt ggt gaa tac   1123
Ala Thr Pro Val Gly Arg Leu Arg Lys Leu Ala Met Gly Gly Glu Tyr
            330                 335                 340
ttg tcg gca ttt acc gta ggt gac cag ttg tta tgg ggc gct gcc gaa   1171
Leu Ser Ala Phe Thr Val Gly Asp Gln Leu Leu Trp Gly Ala Ala Glu
        345                 350                 355
cct ttg cgc aga atg ttg agg att ctg gtc gaa tct taagtaattg        1217
Pro Leu Arg Arg Met Leu Arg Ile Leu Val Glu Ser
    360                 365                 370
tttaagtagc agcccgtaaa gctatgattt atcaataaaa tcatggtctt ttcgggcttt 1277
tgcttttggt gcaatcctgt ttaatggtta ttgtagcctc aaatcctgta tttattgctc 1337
tcaagccgcc tgggtgcgct tgcgtggctg ggtgaatgat gctattttga caaacgccat 1397
gaattactaa gggttaatcg gtgagtaaat ttcaattaaa aaaaatagcc tttgc      1452
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<210> 8
<211> 370
<212> PRT
<213> Methylophilus methylotrophus

<400> 8

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Met Leu Lys Val Gly Phe Val Gly Trp Arg Gly Met Val Gly Ser Val
 1               5                  10                  15
Leu Met Gln Arg Met Met Gln Glu Asn Asp Phe Ala Asp Ile Glu Pro
            20                  25                  30
Gln Phe Phe Thr Thr Ser Gln Thr Gly Gly Ala Ala Pro Lys Val Gly
        35                  40                  45
Lys Asp Thr Pro Ala Leu Lys Asp Ala Lys Asp Ile Asp Ala Leu Arg
    50                  55                  60
Gln Met Asp Val Ile Val Thr Cys Gln Gly Gly Asp Tyr Thr Ser Asp
65                  70                  75                  80
Val Phe Pro Gln Leu Arg Ala Thr Gly Trp Ser Gly His Trp Ile Asp
                85                  90                  95
Ala Ala Ser Thr Leu Arg Met Glu Lys Asp Ser Val Ile Ile Leu Asp
            100                 105                 110
Pro Val Asn Met His Val Ile Lys Asp Ala Leu Ser Asn Gly Gly Lys
        115                 120                 125
Asn Trp Ile Gly Gly Asn Cys Thr Val Ser Leu Met Leu Met Ala Leu
    130                 135                 140
Asn Gly Leu Phe Lys Ala Asp Leu Val Glu Trp Ala Thr Ser Met Thr
145                 150                 155                 160
Tyr Gln Ala Ala Ser Gly Ala Gly Ala Gln Asn Met Arg Glu Leu Ile
                165                 170                 175
Ser Gln Met Gly Val Val Asn Ala Ser Val Ala Asp Leu Leu Ala Asp
            180                 185                 190
Pro Ala Ser Ala Ile Leu Gln Ile Asp Lys Thr Val Ala Asp Thr Ile
        195                 200                 205
Arg Ser Glu Glu Leu Pro Lys Ser Asn Phe Gly Val Pro Leu Ala Gly
    210                 215                 220
Ser Leu Ile Pro Trp Ile Asp Lys Asp Leu Gly Asn Gly Gln Ser Lys
225                 230                 235                 240
Glu Glu Trp Lys Gly Gly Val Xaa Thr Asn Lys Ile Leu Gly Arg Glu
                245                 250                 255
Ala Asn Pro Ile Val Ile Asp Gly Leu Cys Val Arg Ile Gly Ala Met
            260                 265                 270
Arg Cys His Ser Gln Ala Leu Thr Ile Lys Leu Arg Lys Asp Val Pro
        275                 280                 285
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Leu Asp Glu Ile Asn Gln Met Leu Ala Glu Ala Asn Asp Trp Ala Lys
290 295 300
Val Ile Pro Asn Glu Arg Glu Val Ser Met Arg Glu Leu Thr Pro Ala
305 310 315 320
Ala Ile Thr Gly Ser Leu Ala Thr Pro Val Gly Arg Leu Arg Lys Leu
325 330 335
Ala Met Gly Gly Glu Tyr Leu Ser Ala Phe Thr Val Gly Asp Gln Leu
340 345 350
Leu Trp Gly Ala Ala Glu Pro Leu Arg Arg Met Leu Arg Ile Leu Val
355 360 365
Glu Ser
370

<210> 9
<211> 3098
<212> DNA
<213> Methylophilus methylotrophus

<220>
<221> CDS
<222> (1268)..(2155)

<400> 9
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acattattcg cctttgcatg gtaaaggcct atggtcttga tgtaactttc aagacctgcc 120
agccccaaat ccaggatagc ctgcggtgtg ttggccacct tgaacaattt gcgggtggca 180
atattgacac ctttgtctgt cgcctgtgca gacaagatga cggcaatcag taattcgaac 240
gtggagctat gctccagctc agtggttgga ttggggatgg cttgggccag ccgctcaaat 300
atcgccagtc ttttttgtgc attcataaaa cggtttcaat cataggtcac agggtcaacc 360
tgtcttttgc gctttgacgc gcgccatggc tgcggcaatg gcatttttct tgagcacctc 420
agttgagggt gtctcggtcg tagcaagcgt ctggttgcgt ttgctgtagg tttgggcggt 480
ctcccgtttt tcaagggcga ggcgagaaag gcgttgctgg tggcgttgtc tcgctaccgc 540
ggcttcagct tcattcatgg cggtagcccg accgggaatc gtttgcatct gtatgcagtc 600
caccgggcag ggcggtaaac atagctcaca gccagtgcat tcctgggaaa tcaccgtatg 660
catcagtttg gatgcgccca aaatggcatc aacgggacag gcctgtatac acagggtgca 720
gccgatgcat gtttcctcat caatcaaggc caccgctttg ggtttggtga tgccgtgggc 780
cggatttaat gcctggaaag gacgttgcag taatttggca agcgcatgaa tgcccgcttc 840
tcctccaggc ggacattggt tgatattggc ctctccgcgg gcgatcgctt cagcataagg 900

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tttgcatccc tcgtaaccgc attggcggca ttgagtttgc ggtaataccg cgtcgatctt 960
tgcaatgagg tcgacaaagc gttctggcag ctcaggcgca gtcccttcga cttcaatcat 1020
gtgatggcag gtgagtctgc attcggtcct ggctaaatag ccgtttaaga tgggttgcta 1080
agagttttat tataaccgaa accttgcttt tcctttggcc gggagctagg cggaaaaagc 1140
ttgccgcagt tgggtgccag tgattttgcc gccgtcttgc gcttgtatcc gtccagatac 1200
agcaagtagg cgcgttcttt ggcgttagac cggataatca gttaaaatat tcgctttatt 1260
cttaaag atg gcg cta ggt atg tta acg ggc agt ttg gtc gca atc gtg   1309
        Met Ala Leu Gly Met Leu Thr Gly Ser Leu Val Ala Ile Val
          1               5                  10
acc ccc atg ttt gaa gat gga cgt ttg gat ctg gac gcc ctc aaa aag   1357
Thr Pro Met Phe Glu Asp Gly Arg Leu Asp Leu Asp Ala Leu Lys Lys
 15                  20                  25                  30
ctg gtc gac ttt cat gta gag gca ggg aca gat ggt att gtc atc gtt   1405
Leu Val Asp Phe His Val Glu Ala Gly Thr Asp Gly Ile Val Ile Val
                35                  40                  45
ggc acg act ggc gag tcg ccc acg gtg gat gta gat gag cat tgt ctg   1453
Gly Thr Thr Gly Glu Ser Pro Thr Val Asp Val Asp Glu His Cys Leu
            50                  55                  60
ctg atc aaa acc acg atc gag cat gtc gcc aag cgc gtg cca gtc att   1501
Leu Ile Lys Thr Thr Ile Glu His Val Ala Lys Arg Val Pro Val Ile
        65                  70                  75
gcc ggt act ggc gca aat tcc act gct gaa gcc att gaa ctg act gcc   1549
Ala Gly Thr Gly Ala Asn Ser Thr Ala Glu Ala Ile Glu Leu Thr Ala
    80                  85                  90
aag gcc aag gcg ctt ggc gca gac gcc tgc ctg ctg gtg gca ccg tat   1597
Lys Ala Lys Ala Leu Gly Ala Asp Ala Cys Leu Leu Val Ala Pro Tyr
 95                  100                 105                 110
tac aac aag ccc tcg caa gag ggt ttg tac cag cac ttt aaa gcc gtg   1645
Tyr Asn Lys Pro Ser Gln Glu Gly Leu Tyr Gln His Phe Lys Ala Val
                115                 120                 125
gct gag gcg gtc gat att ccg caa att ctc tat aat gtg cca ggc cgc   1693
Ala Glu Ala Val Asp Ile Pro Gln Ile Leu Tyr Asn Val Pro Gly Arg
            130                 135                 140
acc ggt tgc gac ttg tct aac gac acc gta ttg cgc ctg gcg cag att   1741
Thr Gly Cys Asp Leu Ser Asn Asp Thr Val Leu Arg Leu Ala Gln Ile
        145                 150                 155
cgc aac att gtc ggg att aag gat gcg act gga ggg att gag cgc ggt   1789
Arg Asn Ile Val Gly Ile Lys Asp Ala Thr Gly Gly Ile Glu Arg Gly
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    160                 165                 170
acc gat ttg ttg ttg cgt gca cca gct gat ttc gcc att tac agc ggg   1837
Thr Asp Leu Leu Leu Arg Ala Pro Ala Asp Phe Ala Ile Tyr Ser Gly
175                 180                 185                 190
gat gat gcc act gcg ctg gcc ctg atg tta tta ggg ggg aaa ggc gtg   1885
Asp Asp Ala Thr Ala Leu Ala Leu Met Leu Leu Gly Gly Lys Gly Val
                195                 200                 205
att tcg gtc acg gcc aat gtc gcg ccc aaa tta atg cat gaa atg tgc   1933
Ile Ser Val Thr Ala Asn Val Ala Pro Lys Leu Met His Glu Met Cys
            210                 215                 220
gag cat gct ttg aat ggc aac ctg gcc gca gcc aaa gcg gcc aat gcc   1981
Glu His Ala Leu Asn Gly Asn Leu Ala Ala Ala Lys Ala Ala Asn Ala
        225                 230                 235
aaa ctg ttt gca ttg cac cag aag ttg ttt gta gaa gcg aac ccg att   2029
Lys Leu Phe Ala Leu His Gln Lys Leu Phe Val Glu Ala Asn Pro Ile
    240                 245                 250
cca gtg aaa tgg gta tta caa caa atg gga atg att gcc act ggc atc   2077
Pro Val Lys Trp Val Leu Gln Gln Met Gly Met Ile Ala Thr Gly Ile
255                 260                 265                 270
cgt ttg ccg ctg gtc aat tta tcc agc caa tat cat gaa gta ttg cgc   2125
Arg Leu Pro Leu Val Asn Leu Ser Ser Gln Tyr His Glu Val Leu Arg
                275                 280                 285
aac gcc atg aag cag gca gaa att gcc gct tgatcggcta aaactaattt     2175
Asn Ala Met Lys Gln Ala Glu Ile Ala Ala
            290                 295
agggtgaaac aagtgaaata catgagtcat gtttggttac aacgtttggt gctggccagt 2235
ctggtcacag cgctttcagc gtgcgattcc atcccgttta ttgataatag ttctgactac 2295
aagggcgcag gtcgctccag gccacttgaa gtgccgccag acctgaccgc ggtgcgtacc 2355
agcagtactt acaatgtgcc tggtagcacc agttactctg cctatagcca gaaccaggaa 2415
gtgcaagagc agaatggtcc acagcctgtg ctcgcagata tgaaaaacgt gcgcatggtg 2475
aaagcaggcc agcagcgttg gctggtggtc aatgcgcctc cggaaaaaat ctggccgatt 2535
gtgcgtgatt tctggctgga tcaaggcttt gctgtcaggg tagagaatcc tgagcttggc 2595
gtgattgaaa ccgagtggtt gcaatctgat gccatcaagc ctaaggaaga taaccgtggc 2655
tatggtgaaa agtttgatgc ctggctggat aaacttttctg gttttgccga caggcgtaaa 2715
ttccgtacgc gtctggaacg tggggagaaa gacggcacca ccgaaatcta tatgacgcac 2775
cgtactgtcg ccggtgcacc ggatgatggc aaaaattatg tgcagaccca attgggtgtc 2835
attgataccg gttatcgccc caacgcggct gaaaacaaga acaatgccgg taaagagttt 2895
gatgctgact tggatgcaga attactccgt cgaatgatgg tgaaattagg tctggatgag 2955
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cagaaagcag accaggtgat ggcacaatct gcttcagaca agcgtgcaga tgtggtcaag 3015
gagtctgacc agagcgtcac cttgaagttg aatgagccgt ttgaccgtgc ctggcgccgt 3075
gtggcctggc ctggatcccc ggg 3098

<210> 10
<211> 296
<212> PRT
<213> Methylophilus methylotrophus

<400> 10

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Met Ala Leu Gly Met Leu Thr Gly Ser Leu Val Ala Ile Val Thr Pro
 1               5                  10                  15
Met Phe Glu Asp Gly Arg Leu Asp Leu Asp Ala Leu Lys Lys Leu Val
            20                  25                  30
Asp Phe His Val Glu Ala Gly Thr Asp Gly Ile Val Ile Val Gly Thr
        35                  40                  45
Thr Gly Glu Ser Pro Thr Val Asp Val Asp Glu His Cys Leu Leu Ile
    50                  55                  60
Lys Thr Thr Ile Glu His Val Ala Lys Arg Val Pro Val Ile Ala Gly
65                  70                  75                  80
Thr Gly Ala Asn Ser Thr Ala Glu Ala Ile Glu Leu Thr Ala Lys Ala
                85                  90                  95
Lys Ala Leu Gly Ala Asp Ala Cys Leu Leu Val Ala Pro Tyr Tyr Asn
            100                 105                 110
Lys Pro Ser Gln Glu Gly Leu Tyr Gln His Phe Lys Ala Val Ala Glu
        115                 120                 125
Ala Val Asp Ile Pro Gln Ile Leu Tyr Asn Val Pro Gly Arg Thr Gly
    130                 135                 140
Cys Asp Leu Ser Asn Asp Thr Val Leu Arg Leu Ala Gln Ile Arg Asn
145                 150                 155                 160
Ile Val Gly Ile Lys Asp Ala Thr Gly Gly Ile Glu Arg Gly Thr Asp
                165                 170                 175
Leu Leu Leu Arg Ala Pro Ala Asp Phe Ala Ile Tyr Ser Gly Asp Asp
            180                 185                 190
Ala Thr Ala Leu Ala Leu Met Leu Leu Gly Gly Lys Gly Val Ile Ser
        195                 200                 205
Val Thr Ala Asn Val Ala Pro Lys Leu Met His Glu Met Cys Glu His
    210                 215                 220
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Ala Leu Asn Gly Asn Leu Ala Ala Ala Lys Ala Ala Asn Ala Lys Leu
225 230 235 240
Phe Ala Leu His Gln Lys Leu Phe Val Glu Ala Asn Pro Ile Pro Val
245 250 255
Lys Trp Val Leu Gln Gln Met Gly Met Ile Ala Thr Gly Ile Arg Leu
260 265 270
Pro Leu Val Asn Leu Ser Ser Gln Tyr His Glu Val Leu Arg Asn Ala
275 280 285
Met Lys Gln Ala Glu Ile Ala Ala
290 295

<210> 11
<211> 3390
<212> DNA
<213> Methylophilus methylotrophus

<220>
<221> CDS
<222> (2080)..(2883)

<400> 11
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gaagctgcgg gcgaagtgat aaagcagctg aatcaactgt agccactgca agcgacgaat 120
gaaagcaaag gcgctgcact cgctaaggat gaggcagccg aatctcagaa aaccacgtca 180
gagcctgtca aggccgagca agaggtattg ccctcggcca ctgcaacaaa taattcagct 240
gctgcagcga cattggctga agaagaagtg gttccctaca ttccggaggg ggagtatcag 300
gctgcaccca ctccagaaga gatggccaag ggtaatctgg atgtcagtga aaaccaggtt 360
actgaggcta aggcacatcc agtgaatgaa aaggaaatgg ctgcccaaat tgcagatacg 420
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Phe Ser Asp Asn Gly Leu Gln Leu His Ala Ala Leu Asp Arg Ala Glu
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Gly Val Lys Ile Thr Ala Asp Ile His Ala Ala Leu Val Gly Ala Asp
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gtg ctg gtg gat ttc acg cgg ccg gaa gcc agt atg caa tat tta caa    2334
Val Leu Val Asp Phe Thr Arg Pro Glu Ala Ser Met Gln Tyr Leu Gln
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Ile Val Phe Ala Pro Asn Met Ser Val Gly Val Thr Leu Leu Ile Asn
        120                 125                 130
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Leu Val Glu Gln Ala Ala Arg Val Leu Asn Glu Gly Tyr Asp Ile Glu
    135                 140                 145
gtg gtt gaa atg cat cac cgc cat aag gtg gat gcg cct tca ggc acg   2574
Val Val Glu Met His His Arg His Lys Val Asp Ala Pro Ser Gly Thr
150                 155                 160                 165
gct tta cgg ttg ggt gag gct gcg gca aaa ggg att gat aaa gcg ctt   2622
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aaa gat tgt gct gtg tat gcg cgc gaa ggc gtg act ggt gaa cgc gaa   2670
Lys Asp Cys Ala Val Tyr Ala Arg Glu Gly Val Thr Gly Glu Arg Glu
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gcg ggc acg att ggt ttt gca acc tta cgt ggt ggg gat gtg gtc ggt   2718
Ala Gly Thr Ile Gly Phe Ala Thr Leu Arg Gly Gly Asp Val Val Gly
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gac cat acg gtg gtt ctg gct ggt gtg ggt gag cga gta gag tta acg   2766
Asp His Thr Val Val Leu Ala Gly Val Gly Glu Arg Val Glu Leu Thr
    215                 220                 225
cat aaa gca tca agc cgt gcc aca ttt gca caa ggt gcg tta cgt gcg   2814
His Lys Ala Ser Ser Arg Ala Thr Phe Ala Gln Gly Ala Leu Arg Ala
230                 235                 240                 245
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Leu Val Gly Ala Asp Val Leu Val Asp Phe Thr Arg Pro Glu Ala Ser
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Met Gln Tyr Leu Gln Ala Cys Gln Gln Ala Asn Val Lys Leu Val Ile
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Gly Thr Thr Gly Phe Ser Glu Ala Glu Lys Ala Ser Ile Glu Ala Ala
100 105 110
Ser Lys Asn Ile Gly Ile Val Phe Ala Pro Asn Met Ser Val Gly Val
115 120 125
Thr Leu Leu Ile Asn Leu Val Glu Gln Ala Ala Arg Val Leu Asn Glu
130 135 140
Gly Tyr Asp Ile Glu Val Val Glu Met His His Arg His Lys Val Asp
145 150 155 160
Ala Pro Ser Gly Thr Ala Leu Arg Leu Gly Glu Ala Ala Ala Lys Gly
165 170 175
Ile Asp Lys Ala Leu Lys Asp Cys Ala Val Tyr Ala Arg Glu Gly Val
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Thr Gly Glu Arg Glu Ala Gly Thr Ile Gly Phe Ala Thr Leu Arg Gly
195 200 205
Gly Asp Val Val Gly Asp His Thr Val Val Leu Ala Gly Val Gly Glu
210 215 220

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Arg Val Glu Leu Thr His Lys Ala Ser Ser Arg Ala Thr Phe Ala Gln
225                 230                 235                 240
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gcgcttccgc ggcattaccg cttttgatgt aatcctgata gcctggtatg gcgatgctgg 360
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tcaaacgcct caacaagata agtcttcatc gtg acc gct ttt tca atc caa caa  774
                                Val Thr Ala Phe Ser Ile Gln Gln
                                  1               5
ggc cta cta cat gcc gag aat gta gcc ctg cgt gac att gca caa acg  822
Gly Leu Leu His Ala Glu Asn Val Ala Leu Arg Asp Ile Ala Gln Thr
    10                  15                  20
cat caa acg ccc act tac gtc tat tca cgt gcc gcc ttg acg act gct  870
His Gln Thr Pro Thr Tyr Val Tyr Ser Arg Ala Ala Leu Thr Thr Ala
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Phe Ala Val Lys Ala Asn Pro Ser Leu Ala Ile Leu Asn Leu Phe Ala
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cga atg gga gcg ggc ttt gat att gtg tcc ggt ggt gag ctg gca cgc   1014
Arg Met Gly Ala Gly Phe Asp Ile Val Ser Gly Gly Glu Leu Ala Arg
        75                  80                  85
gtc ttg gcc gca ggt ggc gac ccg aaa aaa gtg gtg ttt tct ggt gtg   1062
Val Leu Ala Ala Gly Gly Asp Pro Lys Lys Val Val Phe Ser Gly Val
    90                  95                  100
ggc aaa tcc cat gcg gaa atc aaa gcc gcg ctt gaa gcg ggc att ctt   1110
Gly Lys Ser His Ala Glu Ile Lys Ala Ala Leu Glu Ala Gly Ile Leu
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tgc ttc aac gtg gaa tca gtg aat gag cta gac cgc atc cag cag gtg   1158
Cys Phe Asn Val Glu Ser Val Asn Glu Leu Asp Arg Ile Gln Gln Val
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gcg gcc agc ctg ggc aaa aaa gcg cct att tcc ctg cgc gtg aac ccc   1206
Ala Ala Ser Leu Gly Lys Lys Ala Pro Ile Ser Leu Arg Val Asn Pro
            140                 145                 150
aat gtg gat gcc aaa aca cat ccc tat att tcc cac ccg gct ctc aaa   1254
Asn Val Asp Ala Lys Thr His Pro Tyr Ile Ser His Pro Ala Leu Lys
        155                 160                 165
aac aat aaa ttt ggt gtg gca ttt gaa gat gcc ttg ggc ctc tat gaa   1302
Asn Asn Lys Phe Gly Val Ala Phe Glu Asp Ala Leu Gly Leu Tyr Glu
    170                 175                 180
aaa gcg gcg caa ctg cca aac atc gag gta cac ggc gta gat tgc cat   1350
Lys Ala Ala Gln Leu Pro Asn Ile Glu Val His Gly Val Asp Cys His
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Ile Gly Ser Gln Ile Thr Glu Leu Ser Pro Phe Leu Asp Ala Leu Asp
                205                 210                 215
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            220                 225                 230
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Gln His Ile Asp Val Gly Gly Gly Val Gly Ile Thr Tyr Ser Asp Glu
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Ala Gly Arg Asn Val Lys Val Leu Phe Glu Pro Gly Arg Ala Leu Val
265                 270                 275                 280
ggt aac gcc ggt gtg ctg ctg acc aag gtc gaa tac ctg aaa cct ggc   1638
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                285                 290                 295
gaa acc aaa aac ttt gcg att gtc gat gcc gcc atg aac gac ctc atg   1686
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cgc ccg gct ttg tat gat gct ttc cac aac att acg acc att gcc act   1734
Arg Pro Ala Leu Tyr Asp Ala Phe His Asn Ile Thr Thr Ile Ala Thr
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    330                 335                 340
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Ser Gly Asp Phe Leu Gly His Asp Arg Thr Leu Ala Ile Glu Glu Gly
345                 350                 355                 360
gat tac ctg gcg att cac tcc gca ggc gct tat ggc atg agc atg gcc   1878
Asp Tyr Leu Ala Ile His Ser Ala Gly Ala Tyr Gly Met Ser Met Ala
                365                 370                 375
agc aac tac aac acg cgc gcc cgt gcc gca gag gta ttg gtt gat ggt   1926
Ser Asn Tyr Asn Thr Arg Ala Arg Ala Ala Glu Val Leu Val Asp Gly
            380                 385                 390
gac cag gtg cat gtg atc cgt gaa cgt gaa caa att gcc gac ctg ttt   1974
Asp Gln Val His Val Ile Arg Glu Arg Glu Gln Ile Ala Asp Leu Phe
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Thr Gly His Asp His Leu Ile Cys Phe Ala Val Lys Ala Asn Pro Ser
    50                  55                  60
Leu Ala Ile Leu Asn Leu Phe Ala Arg Met Gly Ala Gly Phe Asp Ile
65                  70                  75                  80
Val Ser Gly Gly Glu Leu Ala Arg Val Leu Ala Ala Gly Gly Asp Pro
                85                  90                  95
Lys Lys Val Val Phe Ser Gly Val Gly Lys Ser His Ala Glu Ile Lys
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Ala Ala Leu Glu Ala Gly Ile Leu Cys Phe Asn Val Glu Ser Val Asn
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Glu Leu Asp Arg Ile Gln Gln Val Ala Ala Ser Leu Gly Lys Lys Ala
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Pro Ile Ser Leu Arg Val Asn Pro Asn Val Asp Ala Lys Thr His Pro
145                 150                 155                 160
Tyr Ile Ser His Pro Ala Leu Lys Asn Asn Lys Phe Gly Val Ala Phe
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Glu Asp Ala Leu Gly Leu Tyr Glu Lys Ala Ala Gln Leu Pro Asn Ile
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Glu Val His Gly Val Asp Cys His Ile Gly Ser Gln Ile Thr Glu Leu
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210 215 220

Leu Ala Ala Lys Gly Ile His Ile Gln His Ile Asp Val Gly Gly Gly
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Val Gly Ile Thr Tyr Ser Asp Glu Thr Pro Pro Asp Phe Ala Ala Tyr
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Thr Ala Ala Ile Leu Lys Lys Leu Ala Gly Arg Asn Val Lys Val Leu
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His Asn Ile Thr Thr Ile Ala Thr Ser Ala Ala Pro Ala Gln Ile Tyr
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Glu Ile Val Gly Pro Val Cys Glu Ser Gly Asp Phe Leu Gly His Asp
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Gly Ala Tyr Gly Met Ser Met Ala Ser Asn Tyr Asn Thr Arg Ala Arg
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## Claims

1. A *Methylophilus* bacterium having L-amino acid-producing ability.

2. The *Methylophilus* bacterium according to claim 1, wherein the L-amino acid is L-lysine, L-valine, L-leucine, L-isoleucine or L-threonine.

3. The *Methylophilus* bacterium according to claim 1, which shows resistance to an L-amino acid analogue or L-amino acid auxotrophy.

4. The *Methylophilus* bacterium according to claim 1, wherein L-amino acid biosynthetic enzyme activity is enhanced.

5. The *Methylophilus* bacterium according to claim 1, wherein dihydrodipicolinate synthase activity and aspartokinase activity are enhanced, and the bacterium has L-lysine-producing ability.

6. The *Methylophilus* bacterium according to claim 1, wherein dihydrodipicolinate synthase activity is enhanced, and the bacterium has L-lysine-producing ability.

7. The *Methylophilus* bacterium according to claim 1, wherein aspartokinase activity is enhanced, and the bacterium has L-lysine-producing ability.

8. The *Methylophilus* bacterium according to any one of claims 5 to 7, wherein an activity or activities of one, two or three of enzymes selected from aspartic acid semialdehyde dehydrogenase, dihydrodipicolinate reductase and diaminopimelate decarboxylase is/are enhanced.

9. The *Methylophilus* bacterium according to claim 5, wherein the dihydrodipicolinate synthase activity and the aspartokinase activity are enhanced by transformation through introduction into cells, of a DNA coding for dihydrodipicolinate synthase that does not suffer feedback inhibition by L-lysine and a DNA coding for aspartokinase that does not suffer feedback inhibition by L-lysine.

10. The *Methylophilus* bacterium according to claim 1, wherein activities of aspartokinase, homoserine dehydrogenase, homoserine kinase and threonine synthase are enhanced, and the bacterium has L-threonine-producing ability.

11. The bacterium according to any one of claims 1 to 10, wherein the *Methylophilus* bacterium is *Methylophilus methylotrophus*.

12. A method for producing an L-amino acid, which comprises culturing a *Methylophilus* bacterium as defined in any one of claim 1 to 11 in a medium to produce and accumulate an L-amino acid in culture and collecting the L-amino acid from the culture.

13. The method according to claim 12, wherein the medium contains methanol as a main carbon source.

14. A method for producing bacterial cells of a *Methylophilus* bacterium with an increased content of an L-amino acid, which comprises culturing a *Methylophilus* bacterium as defined in any one of claim 1 to 11 in a medium to produce and accumulate an L-amino acid in bacterial cells of the bacterium.

15. The method for producing bacterial cells of the *Methylophilus* bacterium according to claim 14, wherein the L-amino acid is L-lysine, L-valine, L-leucine, L-isoleucine or L-threonine.

16. A DNA which codes for a protein defined in the following (A) or (B):

(A) a protein which has the amino acid sequence of SEQ ID NO: 6, or
(B) a protein which has an amino acid sequences of SEQ ID NO: 6 including substitution, deletion, insertion, addition or inversion of one or several amino acids, and has aspartokinase activity.

17. The DNA according to claim 16, which is a DNA defined in the following (a) or (b):

(a) a DNA which has a nucleotide sequence comprising the nucleotide sequence of the nucleotide numbers 510 to 1736 of SEQ ID NO: 5; or
(b) a DNA which is hybridizable with a probe having the nucleotide sequence of the nucleotide numbers 510 to 1736 of SEQ ID NO: 5 or a part thereof under a stringent condition, and codes for a protein having aspartokinase activity.

18. A DNA which codes for a protein defined in the following (C) or (D):

(C) a protein which has the amino acid sequence of SEQ ID NO: 8, or
(D) a protein which has an amino acid sequences of SEQ ID NO: 8 including substitution, deletion, insertion, addition or inversion of one or several amino acids, and has aspartic acid semialdehyde dehydrogenase activity.

19. The DNA according to claim 18, which is a DNA defined in the following (c) or (d):

(c) a DNA which has a nucleotide sequence comprising the nucleotide sequence of the nucleotide numbers 98 to 1207 of SEQ ID NO: 7; or
(d) a DNA which is hybridizable with a probe having the nucleotide sequence of the nucleotide numbers 98 to 1207 of SEQ ID NO: 7 or a part thereof under a stringent condition, and codes for a protein having aspartic acid semialdehyde dehydrogenase activity.

20. A DNA which codes for a protein defined in the following (E) or (F):

(E) a protein which has the amino acid sequence of SEQ ID NO: 10, or
(F) a protein which has an amino acid sequences of SEQ ID NO: 10 including substitution, deletion, insertion, addition or inversion of one or several amino acids, and has dihydrodipicolinate synthase activity.

21. The DNA according to claim 20, which is a DNA defined in the following (e) or (f):

(e) a DNA which has a nucleotide sequence comprising the nucleotide sequence of the nucleotide numbers

1268 to 2155 of SEQ ID NO: 9; or
(f) a DNA which is hybridizable with a probe having the nucleotide sequence of the nucleotide numbers 1268 to 2155 of SEQ ID NO: 9 or a part thereof under a stringent condition, and codes for a protein having dihydrodipicolinate synthase activity.

**22.** A DNA which codes for a protein defined in the following (G) or (H):

(G) a protein which has the amino acid sequence of SEQ ID NO: 12, or
(H) a protein which has an amino acid sequences of SEQ ID NO: 12 including substitution, deletion, insertion, addition or inversion of one or several amino acids, and has dihydrodipicolinate reductase activity.

**23.** The DNA according to claim 22, which is a DNA defined in the following (g) or (h):

(g) a DNA which has a nucleotide sequence comprising the nucleotide sequence of the nucleotide numbers 2080 to 2883 of SEQ ID NO: 11; or
(h) a DNA which is hybridizable with a probe having the nucleotide sequence of the nucleotide numbers 2080 to 2883 of SEQ ID NO: 11 or a part thereof under a stringent condition, and codes for a protein having dihydrodipicolinate reductase activity.

**24.** A DNA which codes for a protein defined in the following (I) or (J):

(I) a protein which has the amino acid sequence of SEQ ID NO: 14, or
(J) a protein which has an amino acid sequences of SEQ ID NO: 14 including substitution, deletion, insertion, addition or inversion of one or several amino acids, and has diaminopimelate decarboxylase activity.

**25.** The DNA according to claim 24, which is a DNA defined in the following (i) or (j):

(i) a DNA which has a nucleotide sequence comprising the nucleotide sequence of the nucleotide numbers 751 to 1995 of SEQ ID NO: 13; or
(j) a DNA which is hybridizable with a probe having the nucleotide sequence of the nucleotide numbers 751 to 1995 of SEQ ID NO: 13 or a part thereof under a stringent condition, and codes for a protein having diaminopimelate decarboxylase activity.

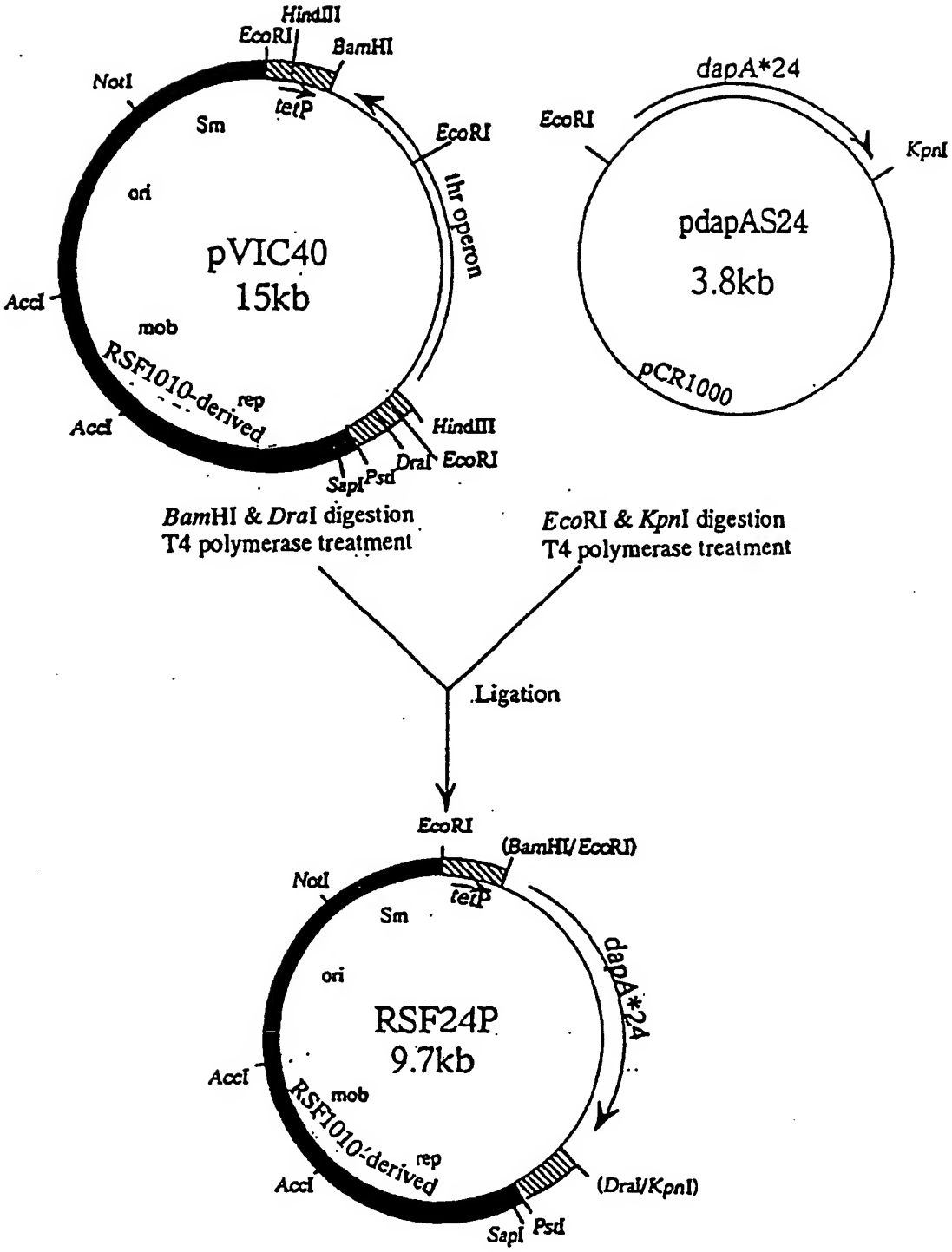
HindIII
EcoRI
BamHI
NotI
tetP
Sm
EcoRI
thr operon
ori
pVIC40
15kb
AccI
mob
RSF1010-derived
rep
AccI
HindIII
DraI
EcoRI
SapI
PstI
dapA*24
EcoRI
KpnI
pdapAS24
3.8kb
pCR1000
BamHI & DraI digestion
T4 polymerase treatment
EcoRI & KpnI digestion
T4 polymerase treatment
Ligation
EcoRI
(BamHI/EcoRI)
NotI
tetP
Sm
dapA*24
ori
RSF24P
9.7kb
AccI
mob
RSF1010-derived
rep
AccI
(DraI/KpnI)
SapI
PstI

FIG. 1

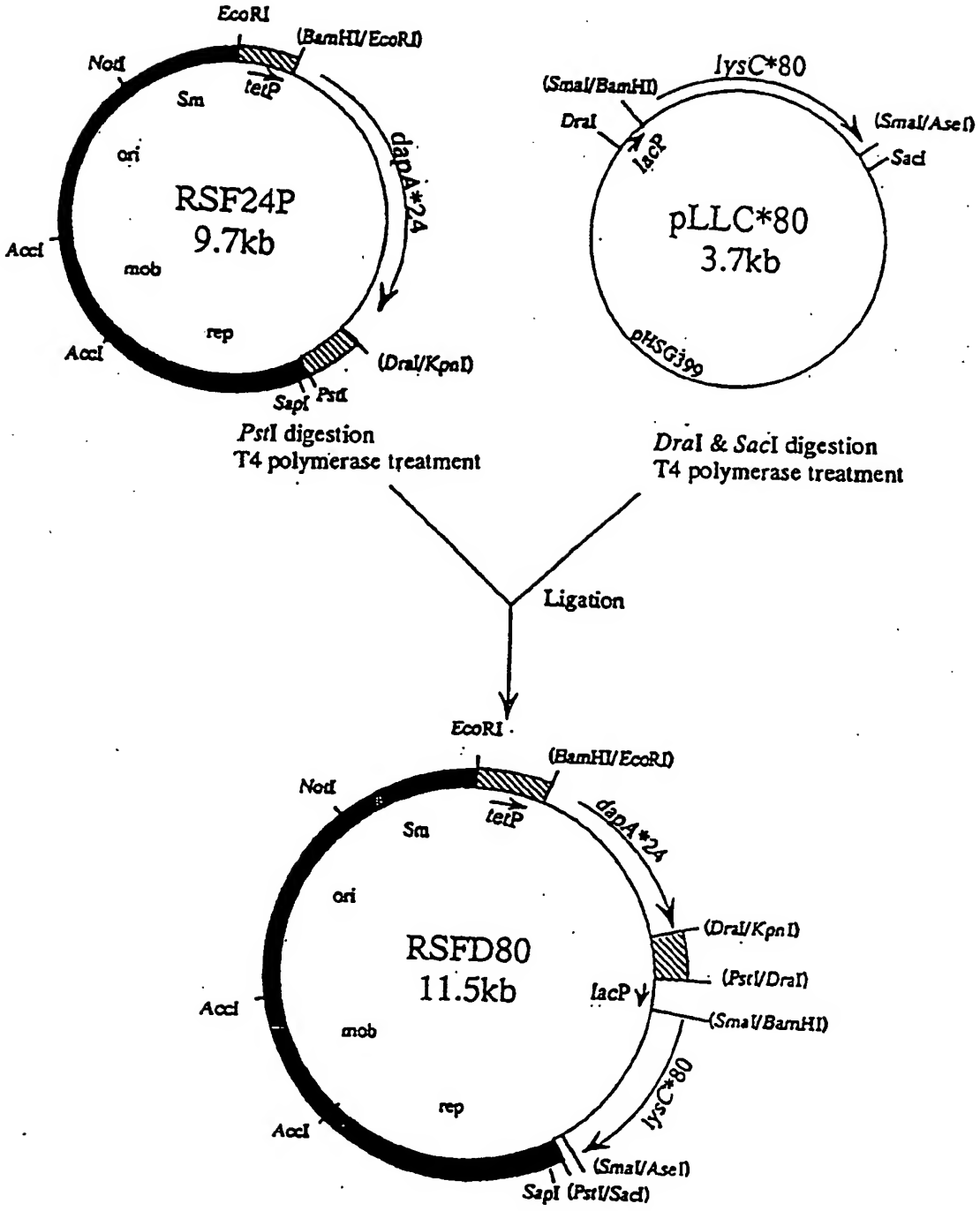
EcoRI
(BamHI/EcoRI)
NotI
Sm
tetP
ori
RSF24P
9.7kb
dapA*24
AccI
mob
rep
AccI
(DraI/KpnI)
SapI
PstI
PstI digestion
T4 polymerase treatment
lysC*80
(SmaI/BamHI)
DraI
lacP
(SmaI/AseI)
SacI
pLLC*80
3.7kb
pHSG399
DraI & SacI digestion
T4 polymerase treatment
Ligation
EcoRI
(BamHI/EcoRI)
NotI
Sm
tetP
dapA*24
ori
(DraI/KpnI)
RSFD80
11.5kb
(PstI/DraI)
lacP
AccI
(SmaI/BamHI)
mob
rep
lysC*80
AccI
(SmaI/AseI)
SapI (PstI/SacI)

FIG. 2

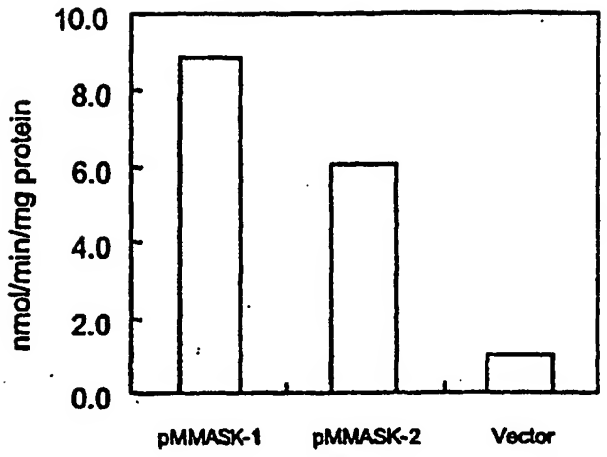
10.0
8.0
6.0
4.0
2.0
0.0
nmol/min/mg protein
pMMASK-1
pMMASK-2
Vector

FIG. 3

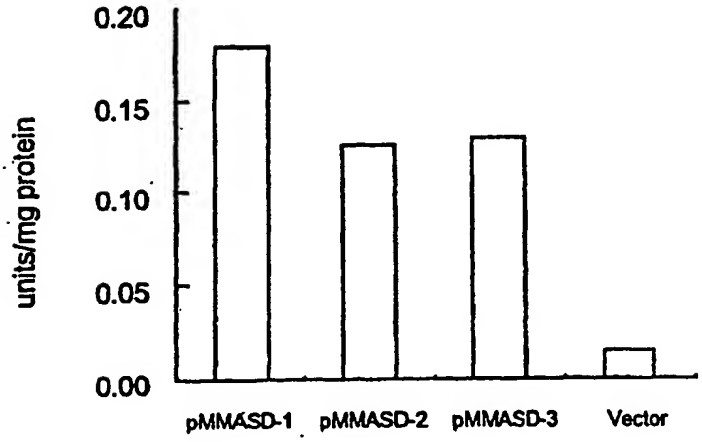
0.20
0.15
0.10
0.05
0.00
units/mg protein
pMMASD-1
pMMASD-2
pMMASD-3
Vector

FIG. 4

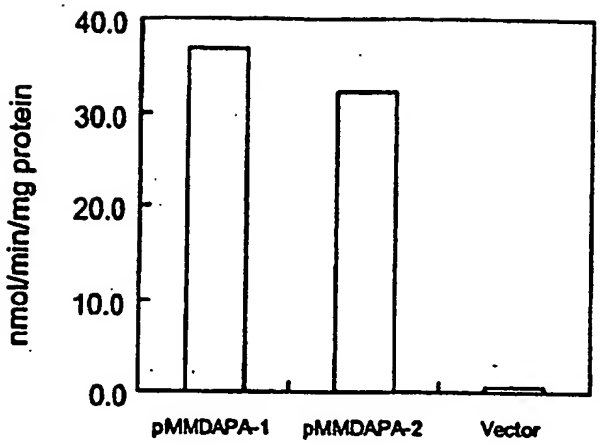
40.0
30.0
20.0
10.0
0.0
nmol/min/mg protein
pMMDAPA-1
pMMDAPA-2
Vector

FIG. 5

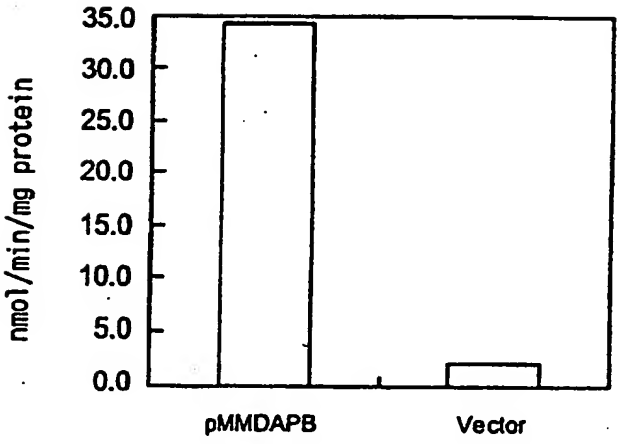
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
nmol/min/mg protein
pMMDAPB
Vector

FIG. 6

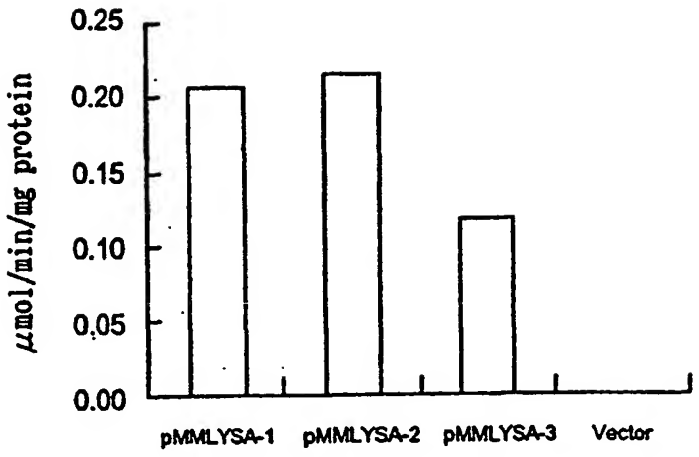
0.25
0.20
0.15
0.10
0.05
0.00
μmol/min/mg protein
pMMLYSA-1
pMMLYSA-2
pMMLYSA-3
Vector

FIG. 7

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/02295

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] C12N1/21, 1/32, 9/00, 15/52, C12P13/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7] C12N1/20-1/21, 9/00-9/99, 15/52-15/61,
C12P13/04-13/14

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
GenBank/EMBL/DDBJ/GeneSeq, SwissProt/PIR/GeneSeq,
WPI (DIALOG), BIOSIS (DIALOG)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | EP, 35831, A2 (IMPERIAL CHEMICAL INDUSTRIES LIMITED), 16 September, 1981 (16.09.81)<br>& NO, 8100773, A & DK, 8100952, A<br>& JP, 56-140893, A & PT, 72630, A<br>& CA, 1187011, A & DE, 3173415, G<br>& RO, 92662, A | 1,4,11-14 |
| X | WINDASS, J. D. et al., "Improved conversion of methanol to single-cell protein by Methylophilus methylotrophus",<br>Nature, October 2, 1980, Volume 287, pp. 396-401 | 1,4,11-14 |
| X | SCHENDEL, Frederick J. et al., "Cloning and Nucleotide Sequence of the Gene Coding for Aspartokinase II from a Thermophilic Methylotrophic Bacillus sp.", Applied and En- vironmental Microbiology, September 1992, Volume 58, Number 9, pages 2806-2814<br>GenBank Accession No. M93419 | 17 |
| X | HOANG, Tung T. et al., "Molecular genetic analysis of the region containing the essential Pseudomonas aeruginosa asd gene encoding aspartate-β-semialdehyde | 19 |

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 05 July, 2000 (05.07.00) | 18 July, 2000 (18.07.00) |
| Name and mailing address of the ISA/<br>Japanese Patent Office | Authorized officer |
| Facsimile No. | Telephone No. |

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/02295

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| | dehydrogenase", Microbiology, March 1997, Volume 143, Part 3, pp. 899-907 GenBank Accession No.U11055 | |
| X | YAMAMOTO, Yoshihiro et al., "Construction of a Contiguous 874-kb Sequence of the Escherichia coli-K12 Genome corresponding to 50.5-68.8 min on the Linkage Map and Analysis of Its Sequence Features", DNA Research, April 28, 1997, Volume 4, Number 2, pp.91-113<br>GenBank Accession No. D90877 | 21 |
| X | BONNASSIE, S. et al., "Nucleotide sequence of the dapA gene from Corynebacterium glutamicum", Nucleic Acids Research, November 11, 1990, Volume 18, Number 21, page 6421<br>GenBank Accession No.X53993 | 21 |
| X | BOUVIER, J. et al., "Nucleotide Sequence and Expression of the Escherichia coli dapB Gene", The Journal of Biological Chemistry, December 10, 1984, Volume 259, Number 23, pp.14829-14834<br>GenBank Accession No. M10611 | 23 |
| X | DEKKERS, Linda C. et al., "A site-specific recombinase is required for competitive root colonization by Pseudomonas fluorescens WCS365", Proceedings of the National Academy of Sciences, USA, June 9, 1998, Volume 95, Number 12, pp.7051-7056<br>GenBank Accession No.Y12268 | 25 |
| A | EP, 37273, A2 (IMPERIAL CHEMICAL INDUSTRIES LIMITED), 07 October, 1981 (07.10.81)<br>& BR, 8101907, A & DK, 8101404, A<br>& JP, 57-8782, A & ZA, 8102086, A<br>& CA, 1187012, A & IL, 62514, A<br>& DE, 3175828, G & KR, 8701127, B | 1-25 |
| A | WO, 96/41871, A1 (Ajinomoto Co., Inc.)<br>27 December, 1996 (27.12.96)<br>& EP, 834559, A1 & SK, 9701705, A3<br>& CN, 1203629, A & HU, 9900149, A2<br>& US, 5989875, A & MX, 9710044, A1 | 1-25 |
| A | Kerney, P. et al., "Regulation and routes of biosynthesis of serine and arginine in Methylophilus methylotrophus ASI", FEMS Microbiology Letters, July 1987, Volume 42, Nos.2-3, pp. 109- 112 | 1-25 |
| A | JP, 1-235595, A (Kyowa Hakko Kogyo Co. Ltd.)<br>20 September, 1989 (20.09.89)<br>(Family: none) | 1-25 |
| A | JP, 53-34987, A (Yoshiki Tani)<br>31 March, 1978 (31.03.78) | 1-25 |

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

## INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/02295

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

The requirement of unity of invention in international application (PCT Rule 13.1) is not satisfied unless there is a technical relationship in a group of inventions involving one or more of the same or corresponding technical features. The term "technical feature" means a technical feature clearly showing the contribution to the prior art by the inventions as set forth in claims as a whole (PCT Rule 13.2). The requirement of unity of invention is judged without considering whether a group of inventions are described in separate claims or in a single claim in an alternative form (PCT Rule 13.3).

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☒ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/02295

Continuation of Box No. II of continuation of first sheet(1)

Inventions as set forth in claims 1 to 25 have a matter in common of a "bacterium belonging to the genus *Methylophilus* and having an L-amino acid productivity". However, document 1 (Japanese Patent Laid-Open No. 140893/1981) and document 2 (Nature, 287(5781), 396-401 (1980)) describe a bacterium belonging to the genus *Methylophilus* and carrying *Escherichia coli*-origin glutamate dehydrogenase (GDH) gene transferred in a state of allowing the expression thereof. Furthermore, a process for producing an amino acid by culturing this bacterium is stated in document 1 (see, for example, claim 19 and thereafter). As also stated in the description (p. 13) of the present international application, GDH gene is a gene imparting an L-glutamic acid productivity to a bacterium belonging to the genus *Methylophilus*. Therefore, it can be said that the bacterium belonging to the genus *Methylophilus* as described in document 1 or document 2 is a "bacterium belonging to the genus *Methylophilus* and having an L-glutamic acid productivity". Accordingly, there had been publicly known a bacterium belonging to the genus *Methylophilus* and having a productivity of l-glutamic acid, i.e., one of L-amino acids. Thus, the "bacterium belonging to the genus *Methylophilus* and having an L-amino acid productivity" which is the matter common to inventions as set forth in claims 1 to 25 cannot be regarded as a "special technical feature" as defined in PCT Rule 13.2.

Also, there had been publicly known a dihydrodipicolinate synthase gene (i.e., a gene capable of imparting an L-lysine productivity to a bacterium belonging to the genus *Methylophilus*) originating in a bacterium belonging to the genus *Corynebacterium* (see, for example, document 3 (Nucleic Acids Res., 18(21), 6421 (1990)). Accordingly, the "special technical feature" common to inventions as set forth in claims 16 to 25 is not an "enzyme gene being usable in enhancing the L-lysine productivity of a bacterium belonging to the genus *Methylophilus*" but an "enzyme gene originating in a bacterium belonging to the genus *Methylophilus* and being usable in enhancing the L-lysine productivity of a bacterium belonging to the genus *Methylophilus*". Thus, it may be said that there is no "special technical feature" as defined in PCT Rule 13.2 between the group of inventions as set forth in claims 1 to 15 relating to a bacterium belonging to the genus *Methylophilus* and having an L-lysine productivity and the group of inventions as set forth in claims 16 to 25.

Such being the case, the claims involve the following six groups of inventions:

① inventions relating to a bacterium belonging to the genus *Methylophilus* and having an L-lysine productivity as set forth in claims 1 to 15;

② inventions relating to a bacterium belonging to the genus *Methylophilus* and having an L-valine productivity as set forth in claims 1 to 15;

③ inventions relating to a bacterium belonging to the genus *Methylophilus* and having an L-leucine productivity as set forth in claims 1 to 15;

④ inventions relating to a bacterium belonging to the genus *Methylophilus* and having an L-isoleucine productivity as set forth in claims 1 to 15;

⑤ inventions relating to a bacterium belonging to the genus *Methylophilus* and having an L-threonine productivity as set forth in claims 1 to 15; and

⑥ inventions as set forth in claims 16 to 25.

Form PCT/ISA/210 (extra sheet) (July 1992)